U.S. DEPARTMENT OF COMMERCE
National Technical Information Service

PB80-116825

Effect of Particulates on Ozone Disinfection of Bacteria and Viruses in Water

Maine Univ at Orono

Prepared for

Municipal Environmental Research Lab, Cincinnati, OH

Aug 79

United States
Environmental Protection
Agency

Municipal Environmental Research
Laboratory
Cincinnati OH 45268

EPA-600/2-79-089
August 1979

Research and Development

PB80-116825

EPA

Effect of Particulates on Ozone Disinfection of Bacteria and Viruses in Water

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U. S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

RESEARCH REPORTING SERIES

Research reports of the Office of Research and Development, U.S. Environmental Protection Agency, have been grouped into nine series. These nine broad categories were established to facilitate further development and application of environmental technology. Elimination of traditional grouping was consciously planned to foster technology transfer and a maximum interface in related fields. The nine series are:

1. Environmental Health Effects Research
2. Environmental Protection Technology
3. Ecological Research
4. Environmental Monitoring
5. Socioeconomic Environmental Studies
6. Scientific and Technical Assessment Reports (STAR)
7. Interagency Energy-Environment Research and Development
8. "Special" Reports
9. Miscellaneous Reports

This report has been assigned to the ENVIRONMENTAL PROTECTION TECHNOLOGY series. This series describes research performed to develop and demonstrate instrumentation, equipment, and methodology to repair or prevent environmental degradation from point and non-point sources of pollution. This work provides the new or improved technology required for the control and treatment of pollution sources to meet environmental quality standards.

This document is available to the public through the National Technical Information Service, Springfield, Virginia 22161.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/2-79-089	2.
3. RECIPIENT'S ACCESSION NO. PB 80 116825	
4. TITLE AND SUBTITLE EFFECT OF PARTICULATES ON OZONE DISINFECTION OF BACTERIA AND VIRUSES IN WATER	5. REPORT DATE August 1979 (Issuing Date)
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Otis J. Sproul, Charles E. Buck, Maura A. Emerson, Douglas Boyce, Douglas Walsh, and Diana Howser	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Civil Engineering University of Maine Orono Orono, Maine 04401	10. PROGRAM ELEMENT NO. 1CC824, SOS 2, Task 10
	11. CONTRACT/GRANT NO. R804587
12. SPONSORING AGENCY NAME AND ADDRESS Municipal Environmental Research Laboratory-Cin.,OH Office of Research and Development U.S. Environmental Protection Agency Cincinnati, Ohio 45268	13. TYPE OF REPORT AND PERIOD COVERED Final 8-20-76 to 1-20-79
	14. SPONSORING AGENCY CODE EPA/600/14

15. SUPPLEMENTARY NOTES

Project Officer: John C. Hoff (513) 684-7331

16. ABSTRACT

This research was initiated in order to determine the effect of particulates on ozone disinfection of enteric bacteria and viruses adsorbed to or incorporated into particulate materials such as fecal material, HEp-2 cells, aluminum oxide floc and bentonite clay. Microorganisms used included fecal coliforms, poliovirus (Sabin Type 1), Coxsackievirus A9, porcine picorna-virus Type 3 (Strain ECPO-6) and f_2 bacteriophage.

The results indicate that the encasement or adsorption of enteric bacteria and viruses in fecal material, both human and porcine, and HEp-2 cells protects these microorganisms from a concentration of ozone and contact time that would normally inactivate the bacteria and viruses in an unadsorbed or free state. HEp-2 cells gave the greatest amount of protection for the cell-associated poliovirus and Coxsackievirus studied. It was necessary to maintain a concentration of 5.33 to 4.81 mg/L ozone to inactivate the cell-associated Coxsackievirus in 5 to 10 minutes. Hydrated aluminum oxide floc and bentonite clay afforded little or no protection to the Escherchia coli, poliovirus and Coxsackievirus adsorbed to these particles over that of the microorganisms in the free state. The f_2 bacteriophage adsorbed to bentonite clay particles was inactivated at a slower rate than the freely suspended phage.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Ozone, Enteroviruses, Polioviruses, Coxsackieviruses, Escherichia coli, Disinfection, Microorganism control (water), Water treatment chemicals, Potable water, Water supply, Turbidity, Particles, Protection	Coliforms	13 B

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) UNCLASSIFIED	21. NO. OF PAGES 89
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE A05-A01

EPA Form 2220-1 (9-73)

U.S. GOVERNMENT PRINTING OFFICE 1979-657-146/5470

EPA-600/2-79-089
August 1979

EFFECT OF PARTICULATES ON OZONE DISINFECTION OF BACTERIA AND VIRUSES IN WATER

by

Otis J. Sproul
The Ohio State University
Department of Civil Engineering
Columbus, Ohio 43210

Charles E. Buck
University of Maine Orono
Department of Microbiology

Maura A. Emerson
Douglas Boyce
Douglas Walsh
Diana Howser
University of Maine Orono
Department of Civil Engineering
Orono, Maine 04401

Grant No. R-804587

Project Officer

John C. Hoff
Drinking Water Research Division
Municipal Environmental Research Laboratory
Cincinnati, Ohio 45268

MUNICIPAL ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

j.a

DISCLAIMER

This report has been reviewed by the Municipal Environmental Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. The complexity of that enviornment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research ; a most vital communications link between the researcher and the user community.

This research provides basic information concerning the disinfection capabilities of ozone, against coliforms and enteric viruses, when these microorganisms are associated with or incorporated into particulate materials. Fecal materials, HEp-2 Cells, aluminum oxide floc and bentonite clay were used as the particulate materials at 1 and 5 NTU.

ABSTRACT

This research program was initiated in order to determine the effect of particulates on ozone disinfection of enteric bacteria and viruses adsorbed to or incorporated into these materials. The particulate materials were fecal material, HEp-2 cells, aluminum oxide floc and bentonite clay. Fecal coliforms, poliovirus (Sabin Type 1), Coxsackievirus A9, porcine picornavirus type 3 (Strain ECPO-6) and f_2 bacteriophage were used in this study. The concentration of particulates to which the bacteria or viruses were adsorbed to or incorporated into before ozonation was 1 or 5 Nephelometric Turbidity Units (NTU).

Results of the ozonation study indicate that the encasement or adsorption of enteric bacteria and viruses in fecal material, both human and porcine, and HEp-2 cells protects these microorganisms from a concentration of ozone and contact time that would normally inactivate the bacteria and viruses in an unadsorbed or free state. The HEp-2 cells gave the greatest amount of protection for the cell-associated poliovirus and Coxsackievirus studied. Equipment was developed to maintain a continuous high ozone concentration in order to inactivate all of the poliovirus and Coxsackievirus incorporated into the cell or cellular material. It was necessary to maintain a concentration of 5.33 to 4.81 mg/L ozone to inactivate the cell-associated coxsackievirus in 5 to 10 minutes. The ozone concentration and the time required to inactivate the virus was far in excess of the ozone concentration necessary to inactivate the unadsorbed or free state virus. Hydrated aluminum oxide floc and bentonite clay afforded little or no protection to the Escherichia coli, poliovirus and Coxsackievirus adsorbed to these particles over that of the microorganisms in the free state. The f_2 bacteriophage adsorbed to bentonite clay particles was inactivated at a slower rate than the freely suspended phage.

This report was submitted in fulfillment of Grant No. R-804587 by the University of Maine Orono under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period August 20, 1976, to January 20, 1979.

CONTENTS

Foreword	iii
Abstract	iv
Tables	vii
Figures	ix
Acknowledgments	x
1. Introduction and Literature Review	1
2. Conclusions	5
3. Materials and Methods	7
Cell Culture	7
LLC-MK2 Cells	7
Minipig Kidney (MPK) Cells	7
HEp-2 Cells	8
Viruses	8
Poliovirus (Sabin Type 1)	8
Coxsackievirus A9	8
Porcine Picornavirus	8
Bacteriophage f_2	8
Virus Stock for Cell-Associated Studies	9
Bacteria	9
Escherichia coli	9
Streptococcus fecalis	9
Fecal *Escherichia coli* Surviving Ozonation	9
Fecal Samples	10
Glassware and Stock Solutions	10
Glassware	10
Solutions	10
Purification of Viruses and Bacteria	11
Poliovirus, Coxsackievirus and Porcine Picornavirus	11
Bacteriophage f_2	11
Escherichia coli	12
Titration of Viruses and Bacteria	12
Poliovirus and Coxsackievirus	12
Porcine Picornavirus	13
Bacteriophage f_2	13
Escherichia coli	14
Streptococcus fecalis	14
Fecal-Associated Microorganisms	14
Preparation of Particulate Materials	14
Hydrated Aluminum Oxide	14
Bentonite Clay	16
Fecal Samples	17
HEp-2 Cell-Associated Virus	17

CONTENTS (Continued)

Presonicated HEp-2 Cell-Associated Virus 18
Microscopy 18
Light Microscopy 18
Ozonation and Disinfection Apparatus 18
Ozonation Apparatus 18
Continuous Flow Disinfection Apparatus 20
Declining Residual Batch Reactor 23
Batch Reactor for Continuous Ozonation 25
Ozone Determination 25
4. Results and Discussion 28
The Effect of Fecal Material on Ozone Disinfection of Coliform Bacteria and Enteric Viruses in Water 28
Results 28
Viruses 28
Bacteria 32
Discussion 36
The Effect of Hydrated Aluminum Oxide Floc on Ozone Disinfection of *E. coli* and Viruses in Water 39
Results 39
Poliovirus 39
Coxsackievirus A9 42
Bacteriophage f_2 42
Escherichia coli 46
Discussion 48
The Effect of Human Epithelial Carcinoma on Ozone Disinfection of Poliovirus (Sabin Type 1) and Coxsackievirus A9 51
Results 51
HEp-2 Cell-Associated Coxsackievirus 54
Batch Reactor 54
Continuous Ozonation of HEp-2 Cell-Associated Polio Virus and Coxsackievirus 54
Discussion 58
The Effect of Bentonite Clay on Ozone Disinfection of *E. coli* and Virus in Water 60
Results 60
Poliovirus 60
Coxsackievirus A9 60
f_2 bacteriophage 60
Escherichia coli 66
Discussion 69

Other Reports Based on this Research 71

References 73

TABLES

Number		Page
1	Concentration of Microorganisms Per Gram of Feces (wet weight)	29
2	Fecal Particle Size Distribution	29
3	Inactivation of Poliovirus (Sabin Type 1) and Fecal-Associated Poliovirus (Sabin Type 1)	30
4	Inactivation of Fecal-Associated Poliovirus (Sabin Type 1) at -20°C	31
5	Inactivation of Porcine Picornavirus Type 3 (Strain ECPO-6) and Fecal-Associated Porcine Picornavirus Type 3 Strain ECPO-6)	33
6	Inactivation of Escherichia coli and Streptococcus fecalis	34
7	Inactivation of Escherichia coli and Fecal-Associated Coliforms	35
8	Inactivation of Escherichia coli (ATCC 15766) and Escherichia coli Surviving Ozonation When Associated with Fecal Material	37
9	Association of Poliovirus (Sabin Type 1) with Hydrated Aluminum Oxide	40
10	Inactivation of Poliovirus (Sabin Type 1) Unadsorbed and Adsorbed at 1 and 5 Nephelometric Turbidity Units (NTU) of Hydrated Aluminum Oxide Floc.	41
11	Association of Coxsackievirus A9 with Hydrated Aluminum Oxide	43
12	Inactivation of Coxsackievirus A9 Unadsorbed and Adsorbed at 1 and 5 NTU of Hydrated Aluminum Oxide Floc	44
13	Association of f_2 Bacteriophage with Hydrated Aluminum Oxide	45
14	Inactivation of f_2 Bacteriophage Unadsorbed and Adsorbed at 1 5 NTU of Hydrated Aluminum Oxide Floc	47
15	Association of Escherichia coli with Hydrated Aluminum Oxide	49

TABLES (Continued)

Number		Page
16	Inactivation of Escherichia coli Unadsorbed and Adsorbed at 1 and 5 NTU of Hydrated Aluminum Oxide Floc.	50
17	Inactivation of HEp-2 Cell-Associated Poliovirus (Sabin type 1) at 1 and 5 NTU.	52
18	Effect of Presonication on Inactivation of HEp-2 Cell-Associated Poliovirus (Sabin Type 1) at 1 and 5 NTU.	53
19	Inactivation of HEp-2 Cell-Associated Coxsackievirus A9 at 1 and 5 NTU.	56
20	Batch Inactivation of HEp-2 Cell-Associated Poliovirus at 5 NTU.	57
21	Batch Inactivation with Continuous Ozonation of HEp-2 Cell-Associated Poliovirus at 5 NTU.	59
22	Association of Poliovirus (Sabin Type 1) with Bentonite Clay	61
23	Inactivation of Poliovirus (Sabin Type 1) Unadsorbed and Adsorbed at 1 and 5 NTU of Bentonite Clay.	62
24	Association of Coxsackievirus A9 with Bentonite Clay	63
25	Inactivation of Coxsackievirus A9 Unadsorbed and Adsorbed at 1 and 5 NTU Bentonite Clay	64
26	Association of f_2 Bacteriophage with Bentonite Clay	65
27	Inactivation of f_2 Bacteriophage Unadsorbed and Adsorbed at 1 and 5 NTU of Bentonite Clay	67
28	Inactivation of Escherichia coli Unadsorbed and in Association with 5 NTU of Bentonite Clay	68
29	Summary of Inactivation of Unadsorbed Organisms	70

FIGURES

Number		Page
1	Ozonation Apparatus	19
2	Continuous Flow Disinfection Apparatus	21
3	Ozonation and Continuous Flow Apparatus	22
4	Batch Reactor With Declining Residual	24
5	Batch Reactor for Continuous Ozonation	26
6	HEp-2 Cells Before Ozonation at 5 NTU	55
7	HEP-2 Cells Sonically Treated Before Ozonation at 5 NTU	55

ACKNOWLEDGMENTS

Following the move of Dr. Sproul, the original Principal Investigator, from the University of Maine to Ohio State University in August 1977, Dr. Charles E. Buck of the Department of Microbiology, University of Maine, assumed responsibility for this research. The efforts of Dr. Buck in assuming this responsibility and seeing the research through to successful completion are gratefully acknowledged.

We also wish to thank Dr. Dean Cliver and Dr. Donn D'Alessio, University of Wisconsin, Madison, Wisconsin for supplying the porcine picornovirus, MPK cell cultures, and porcine feces containing porcine picornovirus (Dr. Cliver) and infant stool specimens containing vaccine type 1 poliovirus (Dr. D'Alessio) for use in this study.

SECTION 1

INTRODUCTION AND BACKGROUND REVIEW

Renovated water may be described as water which has undergone a purification process for the removal of contaminants. Although natural biological and physical processes are capable of renovating water, increased waste loads have necessitated the use of water and wastewater treatment processes to supplement natural renovation processes. At the present time, waste loads are so heavily contaminanted that commonly used technology may not remove the hazards associated with reused water. In addition, decreasing potable water sources may soon require widespread utilization of renovated waste waters for agricultural and domestic purposes, whereas in the past most renovated water was used solely for industrial purposes. Thus, extensive evaluation of the capabilities of purification processes both natural and otherwise are needed to ensure the safety of consumers (1).

The potential hazards associated with direct potable reuse of wastewater have been summarized by Long and Bell (2). One of the primary health threats posed by direct use of renovated water is the transmission of bacterial and viral pathogens. The large number of waterborne disease outbreaks which have been reported in the literature are reasons for concern. Extensive reviews of waterborne outbreaks have been published by Long and Bell (2), Craun et al. (3) and the Committee on Environmental Quality Management (4). Low level transmission of viruses must be considered a source of endemic disease and hence a cause for concern (2).

The majority of waterborne disease outbreaks of known etiology are bacterial in origin. The most commonly identified pathogen during the period between 1971-1974 was Shigella (3). Other genera of pathogenic bacteria found in water as a result of fecal pollution were Salmonella, Vibrio, Mycobacterium, Yersinia and Leptospira (5).

Unlike sporadic outbreaks of waterborne disease caused by bacterial pathogens, illnesses caused by viral transmission in water are difficult to detect. The only viral agent which has definitely been linked to transmission in water is infectious hepatitis virus. However, "any virus excreted in the feces and capable of producing infection when ingested should theoretically be transmissible in water". Therefore, viruses of concern include the enteroviruses (poliovirus, coxsackievirus and echovirus), the adenoviruses and the reoviruses, as well as infectious hepatitis virus (4). In all, more than 100 types of viruses fall into the enteric group and, with the exception of hepatitis virus have been isolated from wastewater and other contaminanted sources of water (6) (7).

The failure of epidemiological studies to implicate water as a route of viral dissemination may result from the fact that small quantities of virus only produce infection rather than disease when ingested (8). Infected individuals, however, excrete viruses in concentrations as high as 10^6 viruses/gm feces (9). Thus, secondary infections may occur and these may result in overt disease (8). Since epidemiological studies normally use disease as the index of pathogen occurrence rather than infection, the role of water in viral transmission may not be apparent.

Viral densities in raw sewage were estimated by Clarke (10) to be about 7000 plaque forming units (PFU)/l. Direct examination of wastewater suggests that densities may actually be somewhat higher than these estimates. As many as 463,500 viruses/liter of raw sewage have been detected in some parts of the world (9), while virus densities in the United States appear to be considerably lower (11).

Despite the high levels of viruses in wastewater, a minimal amount of viruses are present in receiving waters. Commonly used water and wastewater treatment processes will not completely remove viruses (12). Once effluents are discharged to receiving waters, virus dilution, dieoff and other factors combine to reduce virus levels significantly. Reduction of viruses to levels as low as 1 to 2 infectious doses/100 gal may occur (11). These levels of viruses, though difficult to detect, may constitute a health hazard. It has been demonstrated that 1 $TCID_{50}$ (tissue culture infectious dose for 50 percent response) or 2 PFU are capable of producing infection when ingested by gavage (13). Due to the manner in which viruses, particularly enteroviruses, are produced, excreted and transported (intestinal tract→feces→water) and the number and variety of particles in natural waters, it is likely that the majority of viruses in the natural environment are associated with suspended and particulate solids (14). Virus association with solids does not necessarily imply inactivation. Virions may remain infective while associated with solids or they may be eluted from the solids and still retain infectivity (15).

The possibility that bacterial and viral pathogens associated with solids may be protected from disinfectants during treatment and hence remain viable has been a question of considerable importance in recent years. In reviewing the 1962 U.S. Public Health Service Drinking Water Standards, consideration was given to changing the standard for turibidity. The National Interim Primary Drinking Water Regulations established a turbidity Maximum Contaminant Level (MCL) of 1 Nephelometric Turbidity Unit (NTU) with up to 5 units allowed provided the turbidity does not interfere with disinfection, prevent maintenance of effective disinfection throughout the distribution system or interfere with microbiological determinations (16).

The extent of protection given to microorganisms by encasement, adsorption or competitive oxidation of turbidity causing materials, depends on a number of factors. These include the size of the microorganism in question relative to particulate size, the relative innate disinfection resistance of the microorganisms and the type of particle(s) causing turbidity. The importance of these factors are discussed by Hoff (17) in a comprehensive review of the relationship of turbidity to disinfection of potable water.

Hoff concluded that the interference of turbidity with disinfection depends primarily on the type of turbidity present rather than the amount of turbidity. The protection given to microorganisms by inorganic particulate matter is generally negligible or relatively minor. In comparison, organic turbidity offers an appreciable amount of protection to associated microorganisms.

Sproul (12) has indicated that current water and wastewater treatment practices do not effect complete removal of viruses. Berg (8) (14) also maintained that treatment processes constitute adjunctive removal of viruses. In addition, Berg concluded that by removing those substances that interfere with terminal disinfection, treatment processes facilitate the final achievement of total removal or destruction of viruses. In the final analysis, however, reliance for complete destruction of pathogens must be placed on terminal disinfection.

Chlorine traditionally has been the most widely used disinfectant in the United States. Recently, however, its ability to form carcinogenic or potentially carcinogenic compounds such as chloroform and other trihalomethanes has become widely documented (18) (19) (20). As a result of these findings. a review of possible alternate disinfectants has been initiated by the Environmental Protection Agency (18).

Ozone is a possible alternative to chlorine. Although widely used outside the United States, ozone has failed to gain widespread acceptance within the United States. One of the primary reasons for this is the inability of ozone to persist in a distribution system for any length of time. Ozone is a very unstable gas with an effective half life ranging from 20 to 30 minutes. Maintenance of a residual in the distribution system is, therefore, not possible. This problem may be overcome by adding small quantities of a disinfectant, such as chlorine, just before the water passes into the distribution system.

The advantages of ozone far outweigh its disadvantages as a disinfecting agent. Ozone is a potent biocide which does not impart a taste or odor to treated water. To date no obvious effects on the health of consumers from ozonated byproducts have been documented (18). Several extensive reviews of the bactericidal and viricidal capabilities of ozone in water (18) (21) (22) (23) and wastewater (21) (22) treatment have been published. Despite the apparent superiority of ozone, the lack of standardization in disinfection research precludes comparison of most of the data which have been generated.

The principal objective of this investigation was to develop information on the disinfection protential of ozone for viruses and bacteria which have been attached to or incorporated within various solids. Disinfection studies were conducted over a range of O_3 concentrations, using viruses and bacteria in both unadsorbed and adsorbed state, in order to determine whether or not a protective effect was afforded the adsorbed organisms. Experiments were carried out with the solids at 1 and 5 NTU so that a comparison of disinfection data could be made at both turbidity levels. The viruses used for this study were poliovirus (Sabin type 1), coxsackievirus A9 and bacteriophage f_2. In addition, information on the inactivation by ozone of Escherichia coli Hfr K-13 (ATTC#15766) and Streptococcus fecalis (ATTC 19433)

was obtained.
The solids used included fecal materials, aluminum oxide floc, clay and cells.

1. Fecal materials were used to simulate the disinfection condition of wastewater treatment and water treatment plants using a secondary wastewater plant effluent to produce potable renovated water. The naturally occurring fecal associated coliforms, poliovirus (Sabin Type 1) and porcine picornavirus Type 3 (Strain EPCO-6) were used for this study.

2. Hydrated aluminum oxide floc was used to simulate the floc particles formed during the coagulation process used in conventional water and wastewater treatment plants when $Al_2(SO_4)_3$ is used as a coagulant. The viruses used were poliovirus (Sabin type 1), coxsackievirus A9, and f_2 bacteriophage. Inactivation studies were also obtained for Escherichia coli with the aluminum oxide.

3. Bentonite clay suspensions were used because they are natural components of some soils and are known to be an adsorbant of many bacteria and viruses. The viruses used were poliovirus (Sabin type 1), coxsackievirus A9, and f_2 bacteriophage. Escherichia coli was also used in this study.

4. Human epithelial carcinoma cells were used in order to simulate infected and dying cells of the intestinal tract of an individual infected with enteric viruses. These cells may be a source of fecal pollution in a potential drinking water source. Poliovirus (Sabin type 1) and coxsackievirus A9 were used in this study.

SECTION 2

CONCLUSIONS

The results of this study indicate that the encasement or adsorption of enteric bacteria and viruses in fecal material and HEp-2 cells protects these microorganisms from a concentration of ozone that would normally inactivate the bacteria and viruses in an unadsorbed or free state. Protection of fecal-associated coliform bacteria, poliovirus (Sabin Type 1) and porcine picornavirus Type 3 (strain ECPO-6) at 5 Nephelometric Turbidity Unit (NTU) was demonstrated at initial ozone concentrations of 0.096, 0.013 and 0.024 mg/L ozone, respectively. The cell-associated poliovirus and Coxsackievirus at 1 and 5 NTU was not inactivated by ozone concentrations up to 4.68 mg/L ozone for 30 seconds exposure in the Sharp continuous flow disinfection apparatus. Even when the cells were sonicated to break up the clumps of cells and particles, total inactivation was not obtained. However, there was an increase of about one log reduction in the sonicated sample. A batch reactor with decreasing residual and an initial ozone concentration of 3.93 mg/L ozone and an initial titer 1.2×10^5 PFU/ml ozonated stream was also ineffective in inactivating the cell-associated poliovirus at 5 NTU after 75 minutes contact. In the batch reactor with continuous ozonation and initial ozone concentrations of 6.82 and 6.50 mg/L ozone and initial poliovirus concentrations of 2.3×10^5 and 3.6×10^4 PFU/ml respectively the cell-associated poliovirus was inactivated in two minutes. The Coxsackievirus was inactivated in five to fifteen minutes when the initial ozone concentrations were 5.33 to 4.81 mg/L ozone and the initial virus concentrations were 1.9×10^4 to 1.9×10^5 PFU/ml. The initial ozone concentration and residual was somewhat less than used for the poliovirus.

The hydrated aluminum oxide floc offered no protection at either 1 or 5 NTU for poliovirus, Coxsackievirus or E. coli. The inactivation data for adsorbed f_2 bacteriophage were inconclusive since most of the absorbed virus was unrecoverable after being adsorbed to the aluminum oxide floc.

Poliovirus, Coxsackievirus, and Escherichia coli associated with bentonite clay were inactivated by ozone at rates similar to the freely suspended organisms. The inactivation of adsorbed f_2 bacteriophage was slower than the unadsorbed phage. The bentonite clay absorbed virus complex was prepared in concentrations of 1 and 5 NTU for disinfection studies with poliovirus, coxsackievirus and f_2 bacteriophage. Excherichia coli was tested at only 5 NTU.

The particle size of the fecal material, hydrated aluminum oxide floc and bentonite clay was comparable to particulates that may escape water filtration plant. The particle size of the HEp-2 cell-associated virus,

10 to 15 μm, was at the upper limit of size causing turbidity after filtration. Therefore, most of the turbidity particles of HEp-2 cell-associated viruses would have been filtered out before the start of the disinfection process if the water had been filtered.

SECTION 3

MATERIALS AND METHODS

CELL CULTURE

LLC-MK_2 Cells

LLC-MK_2 cells (Hull, Monkey Kidney, Rhesus type) were obtained from (Microbiological Associates, Walkersville, Maryland) and maintained at 37°C in growth medium consisting of Eagle's Minimal Essential Medium (MEM) with Earle's balanced salt solution and L-glutamine (Grand Island Biological Company, Grand Island, New York) containing 2 percent newborn calf serum (Grand Island Biological Company, Grand Island, New York). Antibiotics were routinely incorporated in all media. Sodium penicillin G (The Upjohn Company, Kalamazoo, Michigan), streptomycin sulfate (Eli Lilly and Company, Indianapolis, Indiana) and amphotericin B (E. R. Squibb and Sons, Inc., New York, New York) were employed in the growth medium at levels of 100 units/ml, 100 µg/ml and 5 µg/ml, respectively.

Cells were grown in 32 oz. Brockway prescription bottles (Microbiological Associates, Walkersville, Maryland) until a confluent monolayer was obtained. The monolayer was then dispersed using 0.2 percent trypsin. Approximately 100 ml of fresh medium were added and the suspension was equally divided into 3 bottles.

Minipig Kidney (MPK) Cells

The minipig kidney (MPK) cell line (ATCC CCL166) was propagated at 37°C in growth medium consisting of MEM containing 20 percent fetal calf serum (Grand Island Biological Company, Grand Island, New York), 1 percent essential vitamin mixture (Microbiological Associates, Walkersville, Maryland), 2 percent essential amino acid mixture (Microbiological Associates, Walkersville, Maryland), 300 units/mg penicillin G, 300 µg/ml streptomycin sulfate and 5 µg/ml amphotericin B.

Subcultivation of MPK cells was conducted according to the procedure supplied by Sandy Wolens at the University of Wisconsin, Madison, Wisconsin (Personal Communication). After a monolayer had been obtained in 32 oz. prescription bottles it was washed 3 times with 25 ml aliquots of calcium and magnesium-free phosphate buffered saline (PBS). Ten ml of an enzyme preparation consisting of PBS with 0.01 M sodium ethylenediamine tetraacetate (EDTA) (Fisher Scientific Company, Fair Lawn, New Jersey) and 0.01 percent pancreatin (Grand Island Biological Company, Grant Island, New York) was then added to the flask. After incubating the flask at 37°C for 5 to 10 minutes, the cells were dispersed by aspirating them several times against

side of the bottle. Approximately 60 ml of fresh medium were then added and the cells were equally divided into 2 bottles.

HEp-2 Cells

The HEp-2 cell line was propagated in growth medium consisting of (MEM) with Earle's balanced salt solution, supplemented with 5% mycoplasma tested and virus screened newborn calf serum (Grand Island Biological Company, Grand Island, New York). Penicillin sodium, N.F. (Upjohn Company, Kalamazoo, Michigan) 200 units/ml, streptomycin sulfate, U.S.P. (Eli Lilly and Company, Indianapolis, Indiana) 200 mcg/ml and fungizone (E.R. Squibb and Sons, New York, New York) 5 mcg/ml were routinely added to growth and maintenance media. Cultures were grown in 32 oz. prescription bottles (Microbiological Associates, Walkersville, Maryland) at 37°C.

VIRUSES

Poliovirus (Sabin-Type 1)

Poliovirus (Sabin-Type 1) which had been maintained in the Sanitary Engineering research laboratory at the University of Maine at Orono for several years was used for virus stock development. Virus stock was developed by infecting monolayers of LLC-Mk_2 cells with poliovirus. The growth medium was removed from the monolayer and 5 ml of poliovirus suspension were placed on the cells and allowed to adsorb for 1/2 hours. After adsorption, 40 ml of fresh medium were added and the cells were incubated for approximately 24 hours. When the majority of cells showed evidence of cytopathic effect (CPE), the bottle was shaken to remove the cells. The crude stock was then frozen at -20°C. This procedure was repeated using the crude stock to infect additional cells until a sufficient amount of stock was accumulated.

Coxsackievirus A9

The Coxsackievirus A9 stocks were prepared in the same manner as poliovirus (Sabin type 1). These stocks had also been maintained in the Sanitary Engineering research laboratory at the University of Maine for several years.

Porcine Picornavirus

Porcine picornavirus Type 3 (Strain ECPO-6) was supplied by Dr. Dean Cliver at the University of Wisconsin, Madison, Wisconsin. Monolayers of MPK cells were infected with 5 ml porcine picornavirus and allowed to adsorb for 2 hours. The virus was swirled over the cell sheet every 15 minutes during the adsorption period. Fresh medium was then added and the cells were incubated at 37°C until extensive CPE was noted (usually 2 to 4 days); crude stock was frozen at -20°C.

Bacteriophage f_2

The bacteriophage f_2 (ATCC #15766 B) was obtained from the American Type Culture Collection, Rockville, Maryland. The virus stock was prepared by a

procedure described by Watson and Drewry (24) and Cooper and Zinder (25), and modified by Gentile (26). An aliquot of 100 ml of an 18 hour broth culture of Escherichia coli Hfr K-13 (ATTC #15766) was inoculated into 5 liters of sterile broth. The broth was incubated for 3 hours at 37°C with continuous agitation and aeration. After 3 hours, enough virus was added to yield a 5:1 ratio of virus to E. coli. Incubation for 6 hours at 37°C with agitation and aeration followed. Lysozyme (0.25 mg/ml) was added and incubation continued for 30 minutes. One ml/L of chloroform was introduced, agitated for 30 minutes, and then aerated to remove the residual chloroform. (Both lysozyme and chloroform served to aid lysis).

Virus Stock For Cell Associated Studies

A crude virus stock was prepared by removing the growth medium from confluent monolayers of HEp-2 cell cultures and adding 5 ml of purified Sabin Type 1 poliovirus containing 10^6 plaque forming units (PFU) per ml. After adsorption for 30 minutes, growth medium was replaced. Infected cell cultures were incubated 12 to 18 hours until (CPE) of the monolayer was apparent. The remaining cells were removed by gentle shaking, dispensed to test tubes, and stored at -20°C until use.

Crude virus stocks of Coxsackievirus A9 were obtained in a identical manner. These stocks were stored in test tubes at -20°C until use.

BACTERIA

Escherichia coli

Escherichia coli Hfr K-13 (ATCC 15766) were obtained from the American Type Culture Collection, Rockville, Maryland. The bacteria were grown on nutrient agar (Difco Laboratories, Detroit, Michigan) slants for 24 hours at 37°C. Slants were stored at 4°C and maintained throughout the duration of the project by periodic transferring.

In preparation for an experiment an initial culture of E. coli was prepared by inoculating 100 ml of nutrient broth (BBL, Cockeysville, Maryland) with E. coli from nutrient agar slant. The culture was then incubated 12 to 18 hr at 37°C. Subsequent cultures were prepared by inoculating 100 ml of nutrient broth with 1 ml of a previous broth culture which had been maintained at 37°C.

Streptococcus fecalis

Streptococcus fecalis (ATCC 19433) was processed in a manner similar to that described for E. coli. Trypticase soy agar (BBL, Cockeysville, Maryland) was used in lieu of nutrient agar and trypticase soy broth (BBL, Cockeysville, Maryland) was used in preparations of broth cultures of S. fecalis.

Fecal E. coli Surviving Ozonation

E. coli which survived ozonation were isolated from an EMB agar (Difco

Laboratories, Detroit, Michigan) plate. The identity of the E. coli was confirmed by an IMViC test conducted according to Standard Methods (27).

Fecal Samples

Adult stool specimens were obtained from volunteers at the University of Maine at Orono, Orono, Maine. One gram samples were stored at -20°C in 4 oz. ozone-demand free specimen bottles. These samples were used as a source of fecal-associated coliform bacteria.

Porcine feces containing porcine picornavirus were furnished by Dr. Dean Cliver at the University of Wisconsin, Madison, Wisconsin; samples were stored at -70°C.

Infant stool specimens containing poliovirus (Sabin-Type 1) were supplied by Dr. Donn D'Alessio at the University of Wisconsin, Madison, Wisconsin. Sample titers were determined in the following manner. One-half gram portions of fecal samples were homogenized in 10 ml PBS containing 1500 units/ml penicillin G, 1500 µg/ml streptomycin sulfate and 3 µg/ml amphotericin B by mixing 30 seconds with a Vortex-Genie (Scientific Industries Inc., Bohemia, New York). Samples were then centrifuged 30 minutes at 14,000 x g, 5°C in a fixed angle rotor (Head 870) in a Model B20a IEC refrigerated centrifuge (Damon/IEC Division, Needham Heights, Massachusetts). Supernatant titers were determined. Those samples with the highest titers were pooled for subsequent use; all samples were stored at -20°C.

GLASSWARE AND SOLUTIONS

Glassware

Ozone was used exclusively as the disinfectant in these experiments and glassware associated with sample preparation, adsorption, and ozone determination was treated to make it demand free. Cleaning with a chromic acid solution was followed by 9 rinses with tapwater and 5 rinses with distilled water. The glassware was soaked in distilled water which had been ozonated at a high rate, then dried in a 180°C oven to ensure the elimination of ozone. A cabinet was maintained for the dust free storage of the glassware.

Solutions

Solutions used in these experiments were prepared so as to make them ozone demand free. Distilled water was glass-distilled twice and stored in 20 L carboys. This triple distilled water (TDW) was ozonated at a high rate and solutions were immediately prepared. Excess ozone was allowed to dissipate or was removed by autoclaving the solutions. The pH was maintained at a constant value for all experiments by using TDW which was 0.01 M in NaCl and buffered to pH 7.0 with 0.001 M phosphate from sodium salts. A 20 L volume of this buffer solution was made up for each disinfection trial, and then ozonated to achieve the desired concentration of ozone for the experimentation planned. Following an experiment the remaining buffer was

poured into prescription bottles, autoclaved, and stored. Demand free buffer thus generated was used to dilute virus stock, resuspend samples pelleted by centrifugation, and to carry out adsorption experiments.

PURIFICATION OF VIRUSES AND BACTERIA

Poliovirus, Coxsackievirus and Porcine Picornavirus

Concentration and purification of the crude virus stocks was conducted according to the procedure described by Guskey and Wolff (28) and Boardman and Sproul (29) with minor modifications. Following 3 freeze-thaws, the virus was concentrated tenfold by dialysis against polyethylene glycol 20,000 (PEG) which was obtained from (Fisher Scientific Company, Fair Lawn, New Jersey). The concentrated virus was mixed with an equal quantity of trifluoro-trichloro-ethane (freon) (Polar Chemicals Inc., Lewiston, Maine) and the freon-virus mixture was homogenized to remove cellular debris in a Sorvall Omni-Mxier (Ivan Sorvall Inc., Norwalk, Connecticut) at 14,000 rpm for 2 minutes. The homogenized virus was then centrifuged at 4080 x g, 5°C for 10 minutes in a fixed angle rotor (Head 870) in a Model B20A IEC refrigerated centrifuge. The virus contained in the aqueous phase was further purified by ultracentrifugation onto a 2 to 3 ml cushion of cesium chloride (Sigma Chemical Company, St. Louis, Missouri) containing 0.803 g Cs Cl/ml water. The aqueous phase was ultracentrifuged 2 hours at 131,000 x g, 4°C in an SW 27 swinging bucket rotor in a Beckman Model L5-65 preparative ultracentrifuge (Beckman Instruments Inc., Palo Alto, California). After removing the supernatant, the CsCl cushion was diluted in sterile triple distilled water (TDW) to obtain the desired volume and 1.2 ml aliquots dispensed in 5 ml rubber capped serum vials (Kimble, Toledo, Ohio); stock was stored at -70°C until needed.

Bacteriophage f_2

The bacteriophage f_2 was concentrated and purified according to the techniques described by Watson and Drewry (24) and Cooper and Zinder (25) and modified by Gentile (26). Crude f_2 stock was centrifuged at 5,000 x g for 15 minutes in an SS-34 rotor using a Sorvall RC-2 refrigerated centrifuge (Ivan Sorvall Co. Inc., Norwalk, Connecticut). The supernatant was poured off and saved, the pellet resuspended in 100 ml sterile TDW and homogenized in the Sorval Omni-Mixer for 5 minutes while surrounded by an ice bath. Centrifugation at 5,000 x g for 15 minutes was again initiated and the supernatant was combined with the supernatant obtained from the previous step. Sterile $(NH_4)_2 SO_4$ (0.3 g/ml) was added to the supernatant and the mixture was stored overnight at 4°C to allow complete precipitation. This mixture was then centrifuged at 15,000 x g for 20 minutes and the supernatant discarded. The remaining pellets were washed and consolidated in sterile 28 percent $(NH_4)_2SO_4$ and dissolved in 27 ml of sterile TDW. The dissolved pellets were mixed with a sterile stirring bar for one hour at 4°C. Centrifugation of the mixture followed, and the supernatant was poured off and saved. The pellets were resuspended in 5 ml sterile TDW, disrupted with sonic energy (sonicated), and again centrifuged. The remaining supernatant was combined with that from the previous step. This combined supernatant

was ultracentrifuged at 20,000 x g for 10 minutes in an A-321 rotor in an International B-60 ultracentrifuge. The new supernatant was transferred to fresh centrifuge tubes and ultracentrifuged onto a 2 ml CsCl cushion (0.065 g/ml) at 125,000 x g for 2 1/2 hours at 1°C. The supernatant was then removed from each tube and the CsCl cushions consolidated into two tubes. These tubes were then completely filled with fresh CsCl and ultracentrifuged at 125,000 x g for 20 hours. At this time the fluid was removed from above and below the visible phage bands, and the concentrated phage from both tubes were combined into one tube. Fresh CsCl was again added followed by ultracentrifugation at 125,000 x g for 20 hours. The remaining phage band was diluted 1:400 in sterile TDW and dispersed in 1 ml aliquots into rubber cap serum vials. The phage was then stored at -70°C until needed.

Escherichia coli

Unadsorbed bacterial samples were prepared from 12 to 18 hour broth cultures. The bacteria were harvested by centrifugation at 7,500 x g for 15 minutes in a fixed angle rotor (Head 870) in a model B20A IEC refrigerated centrifuge at 5°C. Soluble ozone demanding material was removed by washing the harvested bacteria. Washings were done by centrifuging the sample at 7500 x g, 5°C for 15 minutes and resuspending the pellet in ozone-demand free 10^{-3} M, pH 7 phosphate buffer with 0.01 M NaCl. After five washings, the final pellet was resuspended in a small amount of buffer. A tenfold dilution of this sample was then made in ozone-demand free buffer. Five ml were injected into the continuous flow apparatus and initial concentrations were then determined.

TITRATION OF VIRUSES AND BACTERIA

Poliovirus and Coxsackievirus

The plague assay procedure of Wolf and Quimby (30) was used for the enumeration of poliovirus and Coxsackievirus. Monolayers of LLC-MK_2 cells were dispersed with 0.2 percent tryspin and suspended in approximately 50 ml of growth medium. One 32 oz bottle was used for every 10 tissue culture plates. After splitting an appropriate number of cell monolayers, the cell suspensions were pooled to obtain a uniform suspension. Five ml portions of the pooled cells were placed in 60 x 15 mm tissue culture plates (Lux Scientific Corporation, Newbury Park, California) and incubated at 37°C in an atmosphere of 5 percent CO_2 until confluent monolayers were obtained.

Tenfold dilutions of poliovirus or Coxsackievirus were made in MEM without serum but with 300 units/ml penicillin G, 300 µg/ml streptomycin sulfate and 5 µg/ml amphotericin B. The growth medium was removed from the plates and 0.2 ml of an appropriate viral dilution placed on duplicate monolayers. The virus was allowed to adsorb for 1/2 hour at 37°C in an atmosphere of 5 percent CO_2. The overlay was prepared by mixing 2 parts of a melted 2 percent purified agar (Difco Laboratories, Detroit, Michigan) which was kept at 50°C, and 3 parts of 2X MEM containing 4 percent newborn calf serum, 300 units/ml penicillin G, 300 µg/ml streptomycin sulfate and 5 µg/ml amphotericin B which was kept at 37°C. The overlay was mixed by inverting several

times and 5 ml of overlay were placed on each plate. The overlay was allowed to harden and the plates were incubated 84-96 hr at 37°C in an atmosphere of 5 percent CO_2 and 100 percent humidity.

Following incubation, the cells were fixed with 37 percent formaldehyde (Fisher Scientific Company, Fair Lawn, New Jersey) for 15 minutes. The overlay was then removed by aiming a jet of water at the agar dish interface. Cell sheets were stained with 1 percent crystal violet in 95 percent ethyl alcohol by transferring the crystal violet from one plate to the next. After staining, the cell sheets were rinsed in a pan with continually running water, dried and counted.

Porcine Picornavirus

Infectivity of the porcine picornavirus was determined by using a microtitration technique. A monolayer of MPK cells from a 32 oz prescription bottle was dispersed as described previously. A 0.2 ml aliquot of medium containing dispersed cells was placed in each well of a microtiter plate (Costar, Cambridge, Massachusetts). The plates were incubated at 37°C in an atmosphere of 5 percent CO_2 until the monolayer reached 50 to 70 percent confluency.

Appropriate serial dilutions of the porcine virus were prepared in MEM as described for propagation of MPK cells, however, no serum was added. The growth medium was removed and a 0.1 ml aliquot of 2X dilution medium was added to each well which was to receive an undiluted sample of porcine picornavirus. A 0.1 ml aliquot of 1X dilution medium was introduced into each remaining well. Replicate 0.1 ml inoculums of each viral dilution were then introduced into six wells. All control wells received an additional 0.1 ml of 1X dilution medium. After incubating the plates 2 hours in an atmosphere of 5 percent CO_2, the medium was removed and replaced with 0.2 ml aliquots of MPK growth medium. Plates were examined 4 to 6 days after infection for CPE. The viral titers were calculated and expressed as 50 percent tissue culture infectious dose ($TCID_{50}$) (31).

Bacteriophage f_2

The procedure for titration of f_2 phage described by Adams (32) and Watson and Drewry (24) was used with minor modifications. Prior to each experiment small overlay tubes were prepared containing 3 ml of 0.7 percent purified agar. Immediately preceding their use, the tubes were autoclaved for 5 minutes and cooled to 50°C. Standard 100 x 15 mm plastic petri dishes (Fisher Scientific Co.), containing nutrient agar (Difco) with 0.85 percent NaCl, were also prepared in advance and stored at 4°C until used. These dishes were warmed to room temperature 1 to 2 hours before needed. Phage samples from particular trials were serially diluted in TDW containing 0.85 percent NaCl. To each overlay tube was added 0.2 ml of an 18 hour nutrient broth (BBL) culture of E. coli followed by 1 ml of diluted virus. After sufficient mixing, the overlay was poured onto the nutrient agar surface of the petri dish and allowed to harden at room temperature for one half hour; two dishes were prepared for each dilution. Incubation at 37°C then

proceeded for 18 to 24 hours at which time the plaques, appearing as clear areas in the bacterial lawn, were counted.

Escherichia coli

Two methods were utilized to determine the concentration of E. coli. Samples which were processed in conjunction with coliforms adsorbed to or encased in fecal material (fecal-associated coliforms) were analyzed by the multiple-tube fermentation technique as described in Standard Methods for the Examination of Water and Wastewater (27). Tenfold dilutions were prepared in saline blanks containing 0.85 percent NaCl. The concentration of E. coli in all other samples was determined by the dilution plate count method; serial dilutions were prepared as indicated above. Duplicate plates containing nutrient agar (Difco) were inoculated with 0.1 ml aliquots of appropriate dilutions and evenly spread over the surface of the plate with a bent, sterilized glass rod. Plates were inverted, incubated 24 hours at 37°C and counted. The concentration of E. coli was expressed as colony forming units (CFU) per 100 ml.

Streptococcus fecalis

Concentrations of S. fecalis was determined by the dilution plate count method. Serial dilutions were prepared in saline blanks containing 0.85 percent NaCl. Plates of trypticase soy agar were prepared in 100 x 15 mm petri dishes (Fisher Scientific Company). Duplicate plates were inoculated as indicated above, incubated 24 hr at 37°C, counted and the concentrations expressed as CFU/100 ml.

Fecal-Associated Microorganisms

Fecal-associated coliform levels were determined by the multiple-tube fermentation technique described previously. Due to the presence of non-coliform bacteria within the fecal samples, primary fermentation tubes demonstrating gas production within 48 hours were all confirmed with 2 percent brilliant green bile lactose broth (Difco Laboratories, Detroit, Michigan).

Titers of fecal-associated porcine picornavirus were determined by microtitration. The titers of poliovirus were determined by plaque assay. These procedures may be found in the section on Virus Titration.

PREPARATION OF PARTICULATE MATERIALS

Hydrated Aluminum Oxide

The adsorbent used was hydrated aluminum oxide, a product of the coagulation process in water treatment when $Al_2(SO_4)_3$ is used as the coagulant. For all microorganisms the procedure used for experimentation was adapted from Boardman and Sproul (29) with various modifications which involved the preparation of adsorbed and unadsorbed samples containing approximately the same number of microorganisms per ml. The adsorption reactor contained 0.01 M NaCl in TDW and sufficient alum as $Al_2(SO_4)_3$ to achieve a final turbidity, after dilution, of 1 or 5 turbidity units, as measured by the Hellige

Turbidimeter (Hellige Inc., Garden City, N.Y.) and defined as a Nephelometric Turbidity Units (NTU), after dilution (see disinfection apparatus) depending on the particular experiment. The concentrations of alum as $Al_2(SO_4)_3$ necessary to obtain these units were 150 and 700 mg/L, respectively. A 10 ml aliquot of virus or bacterial suspension was added to the reaction beaker and the pH simultaneously adjusted to 7.0 by the addition of 0.25 M $NaHCO_3$, yielding a total volume of 500 ml. The beaker was placed under a laboratory stirring machine equipped with two bladed glass stirring rods. The suspension was rapidly mixed for 30 seconds and then gently stirred for 20 minutes at 25 rpm to facilitate floc formation and adsorption of the organisms.

The procedure for sample preparation and adsorption was the same for all three viruses. Virus from the frozen stock was diluted 1:10 in a sterile O_3 demand free buffer solution (0.01 M NaCl in TDW, adjusted to pH 7.0 with 0.25 M $NaHCO_3$). A 10 ml aliquot was extracted for the adsorption procedure as previously described. The 1:10 virus dilution (initial concentration) was then further diluted 1:50 so that an unadsorbed sample (for disinfection) might be obtained containing approximately the same virus concentration as that taken from the adsorption reactor (10:500). The adsorption process then proceeded as previously described for 20 minutes. Five 40 ml samples were withdrawn for centrifugation at 1,900 x g for 30 minutes at 5°C in a fixed angle rotor (head 870) using a Model B 20 A IEC refrigerated centrifuge, in order to separate the unadsorbed virus from the floc particles and its adsorbed fraction. The supernatant was then poured off and titrated so that the extent of viral adsorption could be calculated by subtracting the supernatant titer from the initial titer. The pellets were then resuspended in the buffer solution mentioned before. This resuspension was also titrated in order to determine the actual recoverable adsorbed fraction. A 160 ml sample was then homogenized in a Sorvall Omni-Mixer at 4,000 rpm for 30 seconds in order to obtain a floc particle size range that was advantageous for experimentation. An aliquot was withdrawn from this volume to be utilized as the adsorbed sample for disinfection. Preliminary studies were conducted to determine the concentration levels of alum as $Al_2(SO_4)_3$ necessary to achieve a final turbidity, after homogenization and dilution, of 1 or 5 NTU. Another 40 ml portion was extracted and recentrifuged to determine that fraction of virus released by the homogenization process (obtained from the supernatant titer). A titer of the homogenized sample was also taken to determine the amount of virus that actually remained adsorbed following homogenization. A control reactor, without the alum, was also maintained throughout the adsorption and centrifugation steps to determine the loss of virus attributed to experimental procedure. This was accomplished by titrating the control sample after it was centrifuged.

For the bacterial preparation, an 80 ml volume of an 18 hour *E. coli* broth culture was centrifuged at 5°C for 15 minutes at 7,500 x g in a fixed angle rotor (head 870) using a Model B 20 A IEC refrigerated centrifuge. The pellets were combined into one and washed five times in buffer solution at the above speed and time duration. The washed pellet of *E. coli* was then resuspended in approximately 50 ml of buffer and a 10 ml sample withdrawn for adsorption as previously stated. This bacterial suspension was then diluted 1:50, as described for the viruses, in order to match the unadsorbed bacteria

count with that of the adsorbed. A 10 ml aliquot of this dilution was used for disinfection of unadsorbed bacteria.

A settling procedure was used to separate the floc adsorbed E. coli from the unadsorbed E. coli. The large particle size of E. coli caused the unadsorbed bacteria to pellet with those adsorbed on the floc particles at the centrifuge speeds necessary to completely pellet the hydrated aluminum oxide floc. An aliquot taken from the adsorption beaker and assayed to determine the recovery of bacteria from the floc, therefore, contained the unadsorbed bacteria. The 160 ml sample taken from the adsorption beaker for homogenization, and the aliquot withdrawn and plated after homogenization to determine the recoverable bacteria after completion of this process also contained unadsorbed E. coli. Therefore, in order to determine the actual extent of adsorption, the remaining volume in the adsorption beaker was settled at 4°C. Complete settling of the floc required to obtain 1 NTU was approximately 45 minutes. The floc used to obtain 5 NTU, however, required 24 hours for complete segregation. After settling, an assay of the supernatant was taken to determine the concentration of unadsorbed bacteria. The volume remaining after sample homogenization (both 1 and 5 NTU) was also allowed to settle for 24 hours at 4°C so that the concentration of bacteria released by homogenization could be determined from the supernatant count. At the same time a control reactor with E. coli but without alum was set up at 4°C for 24 hours to determine the natural die-off of E. coli after 24 hours. This also permitted determination of the sedimentation of unadsorbed E. coli which would occur naturally in this time span. Correction factors for both phenomena were applied to the supernatant concentrations obtained above. The actual concentration of bacteria recovered from the floc was then calculated by subtracting the corrected value obtained for the supernatant in the adsorption beaker from the concentration of the previously assayed sample that contained both adsorbed and unadsorbed E. coli. In the same manner, the concentration of recoverable bacteria after homogenization was calculated by subtracting the corrected homogenized supernatant concentration from the homogenized sample concentration that contained both adsorbed and unadsorbed bacteria. As was the case for viruses, an aliquot of 10 ml of the homogenized mixture was utilized for disinfection of the adsorbed bacteria with a control reactor used without alum to determine the losses due to experimental procedure.

Bentonite Clay

Bentonite clay B-235 (Fisher Scientific Co.) was used to represent the behavior of naturally occurring particulates. A stock suspension of discrete particles was prepared by adding clay powder to a Waring blendor containing 1 L of TDW and blending until a homogeneous mixture was obtained. The mixture was poured into a large carboy containing 48 L of TDW equipped with a stirring device to evenly disperse the clay particles. After a 3 hour quiescent settling period, 8 L of clay stock were removed via a siphon suspended 1/3 the height of the carboy above the base. Turbidity of the siphoned fraction was determined with a Hellige Turbidimeter and the particles were measured with a microscope that had been calibrated with a stage micrometer. The clay stock was transferred to a 20 L carboy and ozonated at a high rate for sufficient time to make it demand free. Disinfection studies

of the clay-associated viruses were devised to produce reaction tube particulate concentrations of 1 and 5 NTU as measured with a Hellige Turbidimeter (Hellige, Inc., Garden City, NY). The 5 NTU level was found to correspond to a bentonite concentration of about 35 mg/L.

Adsorption of the viruses to the clay was accomplished through modifications of a procedure developed by Boardman and Sproul (29). A given amount of prepared bentonite was added to a one L beaker containing an appropriate volume of buffer solution. A concentrated solution of the phosphate buffer system was added to adjust for the addition of the clay stock. The virus formed by the dilution of the thawed virus concentrate was introduced and the beaker was placed under a laboratory sitrring machine. Using 2 bladed glass stirrers, the suspension was mixed at 100 rpm for 30 seconds and then gently stirred at 25 rpm for a specified period to allow for adsorption. For f_2 phage, poliovirus and coxsackievirus, 30 minutes were allowed for adsorption. At the end of the adsorption period, a 40 ml sample was centrifuged at 1800 x g for 30 minutes in the case of f_2, and 20 minutes for the poliovirus and coxsackievirus.

The supernatant fluid was decanted and analyzed for virus. The pellet was resuspended in buffer solution and analyzed for adsorbed virus since the virus associated with the particulate matter would have been removed by centrifugation.

In the case of E. coli, an adsorption period of 20 minutes was allowed. The mass of the bacteria prevented separation of the unadsorbed organisms from the bentonite particles by centrifugation, thus no adsorption data were generated for E. coli.

Fecal Samples

A modification of the procedure supplied by Dr. John C. Hoff, Drinking Water Research Division, Environmental Protection Agency, Cincinnati, Ohio was used in the preparation of fecal samples (Personal Communication). One gram of fecal material was suspended in 100 ml ozone-demand free pH 7.0 phosphate buffer by homogenizing it in a Sorval Omni-Mixer for 1/2 minute at 11,500 rpm. The suspension was allowed to settle overnight. After settling overnight, the supernatant was centrifuged and the pellet washed in the manner described above for the unadsorbed bacteria. The final pellet was resuspended in a small quantity of ozone-demand free phosphate buffer and the turbidity determined using the Hellige Turbidimeter. The sample was then diluted to give a 10 ml sample with a turbidity of either 29 or 145. Further dilution of these samples within the disinfection reactor yielded turbidities of 1 and 5, respectively. Five ml were injected into the disinfection apparatus. Initial levels of viruses and coliforms were measured on the samples before injection.

HEp-2 Cell-Associated Viruses

The procedure of Symons and Hoff (16) was used to obtain cell-associated viruses. Confluent monolayers of HEp-2 cell cultures were infected with 20 to 40 PFU/cell of either poliovirus or coxsackievirus as previously described.

Infected cell cultures were incubated 12 to 15 hours at 37°C until CPE was just visible. Cells were removed by gentle shaking and the resultant cell suspension centrifuged 15 minutes at 2,000 rpm in an IEC B-20A refrigerated centrifuge. The supernatant was discarded. The pellet was washed five times by repeated centrifugation of 2,000 rpm for 15 minutes. A stock cell suspension was prepared by resuspending the pellet in 15 ml of ozone demand free PH70 phosphate buffer in .01 M NaCl so that its turbidity would produce a final turbidity of either 1 NTU or 5 NTU after dilution in the reaction container. A portion of the stock cell suspension was reserved to determine the titer of the cell-associated virus before ozonation.

Presonicated HEp-2 Cell-Associated Virus

A comparison of the inactivation of HEp-2 cell-associated poliovirus was performed utilizing duplicate portions of a sample, one of which had been sonically treated. The initial sample was divided, and one aliquot was treated 30 seconds at 50 kilocycles/sec with a sonic dismembrator (Fisher Scientific, Fair Lawn, N.J.). The susceptibility to inactivation by ozone of the sonically treated cell-associated poliovirus and cell-associated virus not exposed to sonication was determined at various applied ozone dosages in the Sharp disinfection apparatus.

MICROSCOPY

Light Microscopy

Photo micrographs were obtained utilizing a 35 mm camera attached to a Phase Star, Spencer microscope (American Optical, Buffalo, New York). Replicate samples of presonicated HEp-2 cell-associated poliovirus and HEp-2 cell-associated poliovirus which had not been sonically treated were withdrawn after 10 seconds, 20 seconds, and 30 seconds exposure to ozone in the Sharp continuous flow apparatus. The physical state of the remaining cellular material at each contact time was compared to cellular material of the initial samples which were not exposed to ozone. Initial samples were diluted with ozone demand free buffer to a turbidity of 5 NTU to faciliate comparison with samples obtained from the Sharp disinfection apparatus.

OZONATION AND DISINFECTION APPARATUS

Ozonation Apparatus

The ozonation apparatus was constructed as shown in Figure 1. Ozone was generated from an extra dry grade oxygen with a Model SG 4060 ozone generator (Union Carbide Corporation, South Plainsfield, New Jersey). Oxygen was fed through a two-stage regulator at a working pressure of 50-100 psig.

A small amount of oxygen was diverted before reaching the ozone generator and mixed with ozone entering the 20 L Pyrex contact vessel. The oxygen served to increase turbulence within the vessel, thus promoting the transfer of ozone into the liquid. Diffusion of the gas mixture was accomplished by a Corning immersion tube (Fisher Scientific Company, Medford,

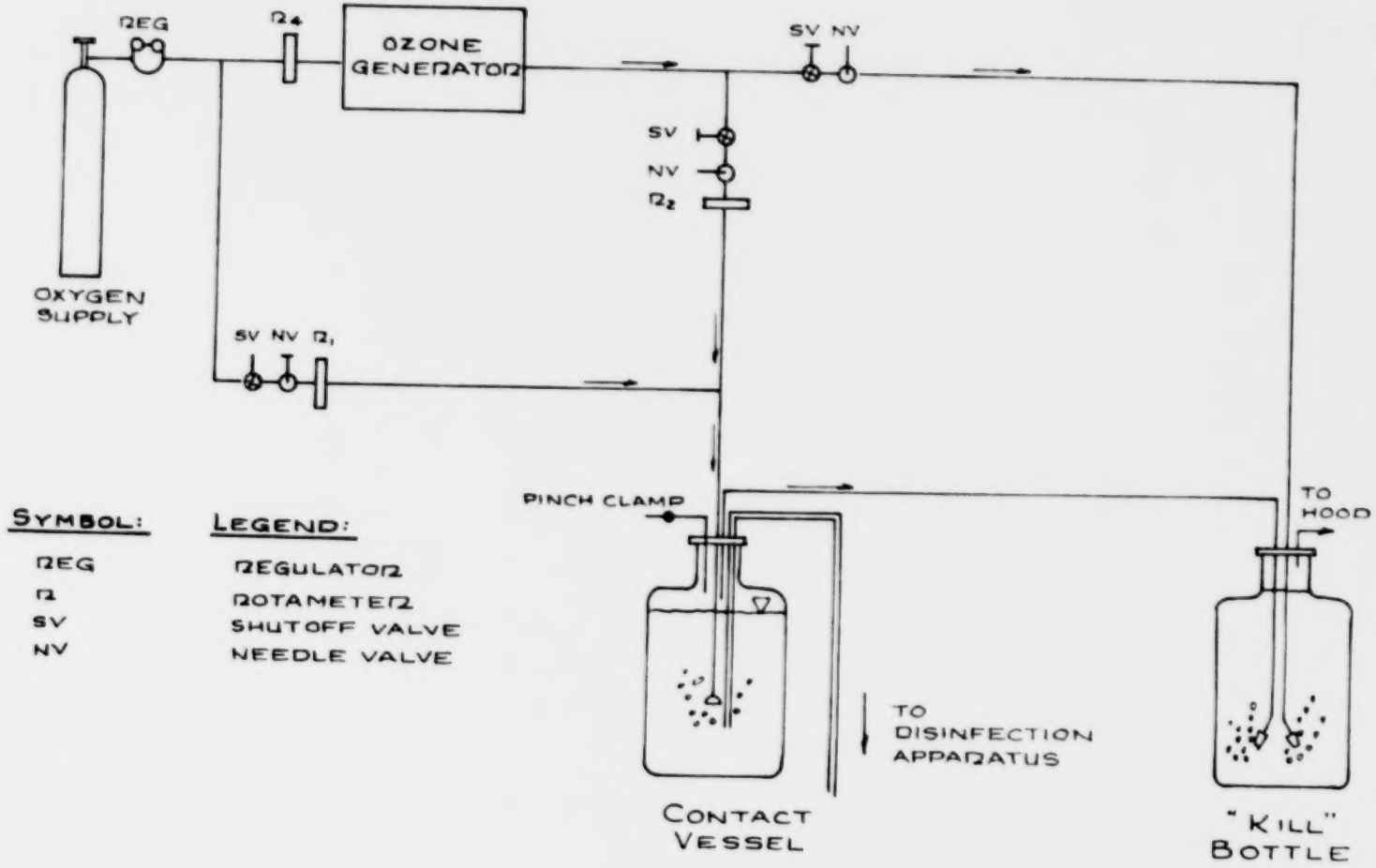

Figure 1. Ozonation Apparatus

Massachusetts).

Since low levels of ozone were required for inactivation experiments, a large portion of the ozone generated was vented directly to a kill bottle. Neutralization of the excess ozone was accomplished by 2.5 kg sodium thiosulfate and 50 g potassium iodide in approximately 20 L of tap water. Aquarium diffusers were used in the kill bottle.

To minimize the ozone demand of the system, 316 grade stainless steel (SS) tubing (1/4" O.D. x .035" wall) was used. The tubing was coupled with 316 grade SS Swagelok fittings. Diffusers and vents in the contact vessel and kill bottle were made of glass. Sections of glass and stainless steel were mated with Tygon tubing.

Continuous Flow Disinfection Apparatus

Experiments were conducted using the Sharp continuous flow disinfection apparatus (36) as shown diagrammatically in Figure 2. The equipment is shown in the photograph in Figure 3. Modifications were made as necessary to accommodate ozone as the disinfectant.

The reaction tube was made of 316 grade stainless steel (1/2" O.D. x .035" wall) coupled with Swagelok 316 grade stainless steel unions. Holes drilled through the center nuts of the Swagelok unions served as access ports; these ports were covered by disposable, rubber, serum vial stoppers (Arthur H. Thomas Company, Philadelphia, Pennsylvania). Access ports were spaced 10 feet apart. The first port served as the sample injection port. A 10 mesh stainless steel wire grid was placed just downstream of the injection port to aid in mixing. Upstream of the injection port, a Whitey regulating valve was installed to control flow. Four access ports downstream of the sample injection port were utilized for sample withdrawal. These ports were equipped with a solenoid coils (Sporlan Valve Company, St. Louis, Missouri) which surrounded the sampling syringes. The tubing outflow was open to the atmosphere.

The apparatus was designed to operate under a siphon head. Since it was essential to maintain turbulent flow in the reaction tubing a Reynolds number greater than or equal to 3000 was maintained. Since

$$N_R = \frac{Vd}{\nu} ,$$

where:

v = velocity
d = diameter = 0.036'
ν = kinematic viscosity
= 1.09×10^{-5} ft^2/sec @ 20^{0}C,

a velocity greater than or equal to 0.9 fps was required. For convenience, a velocity of one foot per second (fps) was selected and by $Q = VA$ a flow requirement was established. The height of the contact vessel was constantly

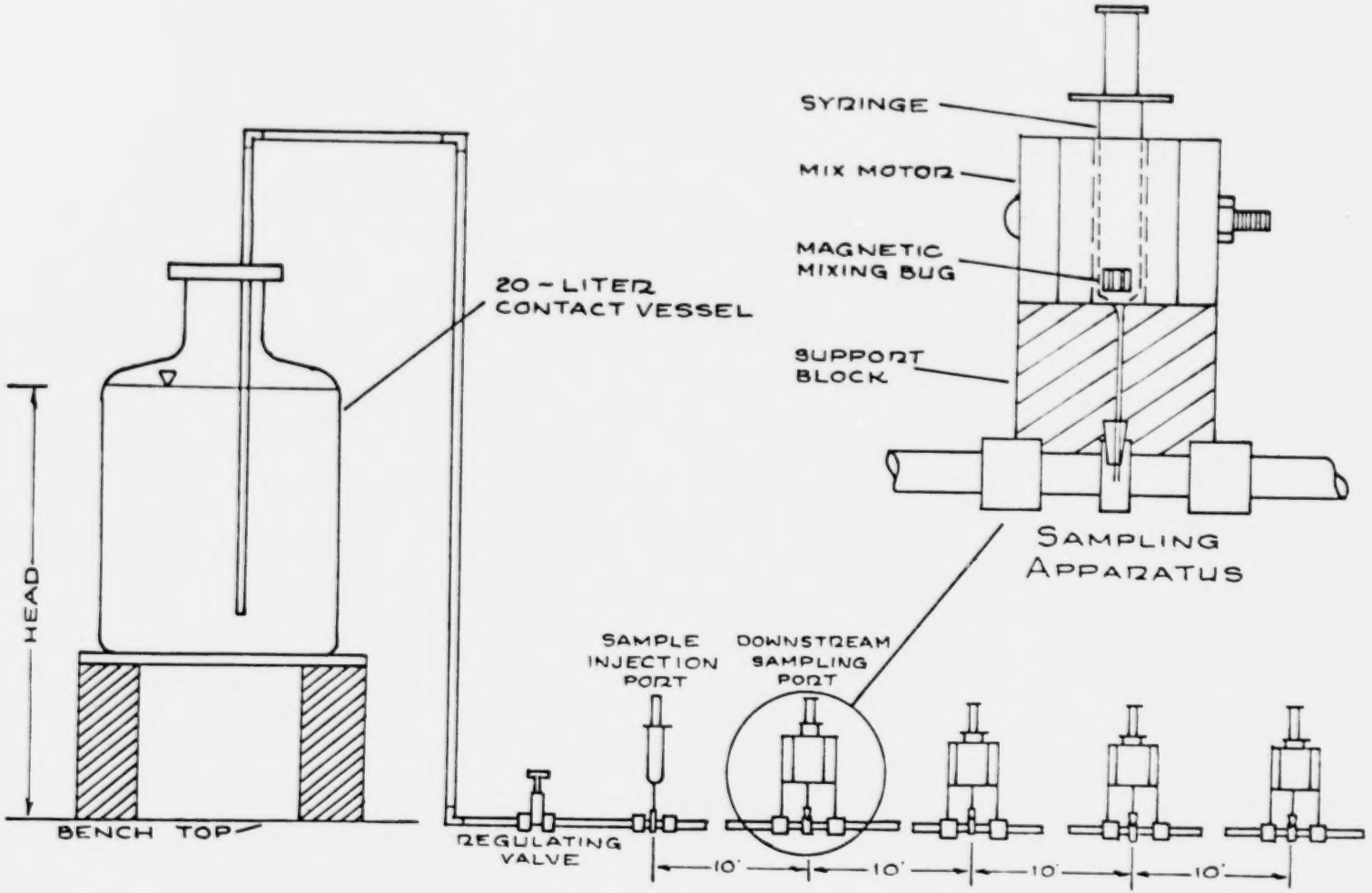

Figure 2. Continuous Flow Disinfection Apparatus

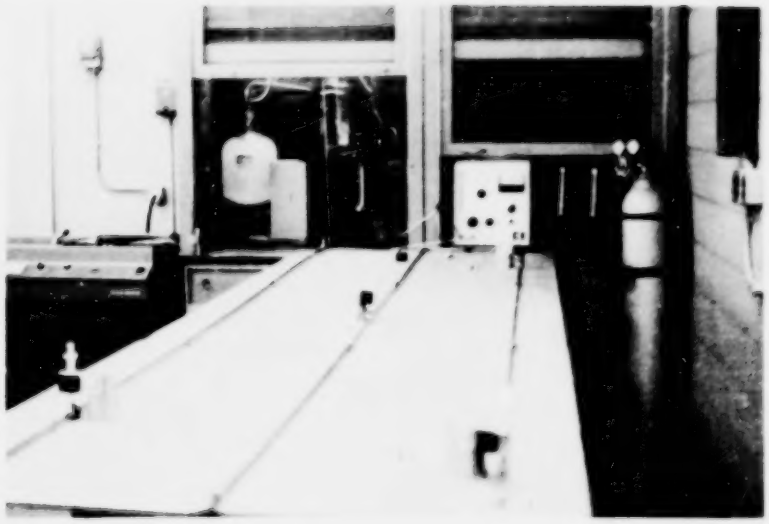

Figure 3. Ozonation and continuous flow apparatus

maintained at a level that yielded a flow greater than that required throughout the course of an experiment. A metering valve was utilized to decrease the flow to an average velocity of one fps when the volume in the contact vessel ranged from 20 L to greater than 14 L. It was possible to maintain this average velocity because of the large cross sectional area of the contact vessel. With a velocity of one fps and a distance of 10 feet between access ports the time of transit between ports was 10 seconds. Thus, samples for analysis were taken at contact times of 10, 20, 30, and 40 seconds.

Sample injection was accomplished in the following manner. A 5 ml sample of organisms was injected manually into the ozonated stream utilizing a 5 ml syringe. The sample was added over a 5 second time period at a precise rate of 1 ml/second.

Samples for analysis were extracted manually at downstream sampling ports. Since samples were injected over 5 seconds, a five second time period was available for sampling. To smooth out any inaccuracies in the manual addition of the organisms, samples were taken during the second, third, and fourth seconds. The samples were withdrawn utilizing 5 ml syringes with 22 gauge needles. Each syringe contained 1 ml of 0.025 N sodium thiosulfate and a 3/8" x 5/16" Nalgene star magnetic mixer (Arthur H. Thomas Company, Philadelphia, Pennsylvania). Residual ozone was neutralized by continually mixing the sample with thiosulfate. The mixers were agitated by valve solenoids surrounding the sample syringes.

Injection of a 5 ml sample of organisms into the reaction tubing over a 5 second period yielded a dilution factor of 29 to 1. The dilution factor was verified by injecting organisms into unozonated dilution water and withdrawing samples downstream at 10, 20, and 30 seconds.

In a typical experiment the contact vessel was filled with 20 L of a 0.01 M NaCl solution prepared utilizing triple distilled water (TDW). The solution was adjusted to 20^{0}C and buffered to yield 10^{-3} M, pH 7 phosphate. The buffer was then ozonated to achieve the desired concentration. The ozonated buffer was siphoned through the reaction tubing at the desired flowrate. Triplicate samples were taken at the outfall to determine ozone residual and a 10 ml sample of buffer was taken at the 40 second sample port to aid in determining ozone demand of the samples. (An 18 gauge needle was necessary to obtain a 10 ml sample in the alloted sampling period). Two samples were then injected into the reaction tubing. The first sample contained the unadsorbed virus or bacteria. Approximately one minute after injecting the first sample, a sample of virus or bacteria in an adsorbed state was introduced into the reaction tubing. Samples for analysis were taken at contact times of 10, 20, and 30 seconds. Ten ml samples were taken at the 40 second sample port for determination of ozone demand. All samples taken at 40 seconds were immediately transferred to ozone-demand free test tubes; samples were then assayed for survival.

Declining Residual Batch Reactor

The batch reactor with a continually declining ozone residual was assembled as shown in Figure 4. This reactor was made from a 500 ml Erlenmeyer

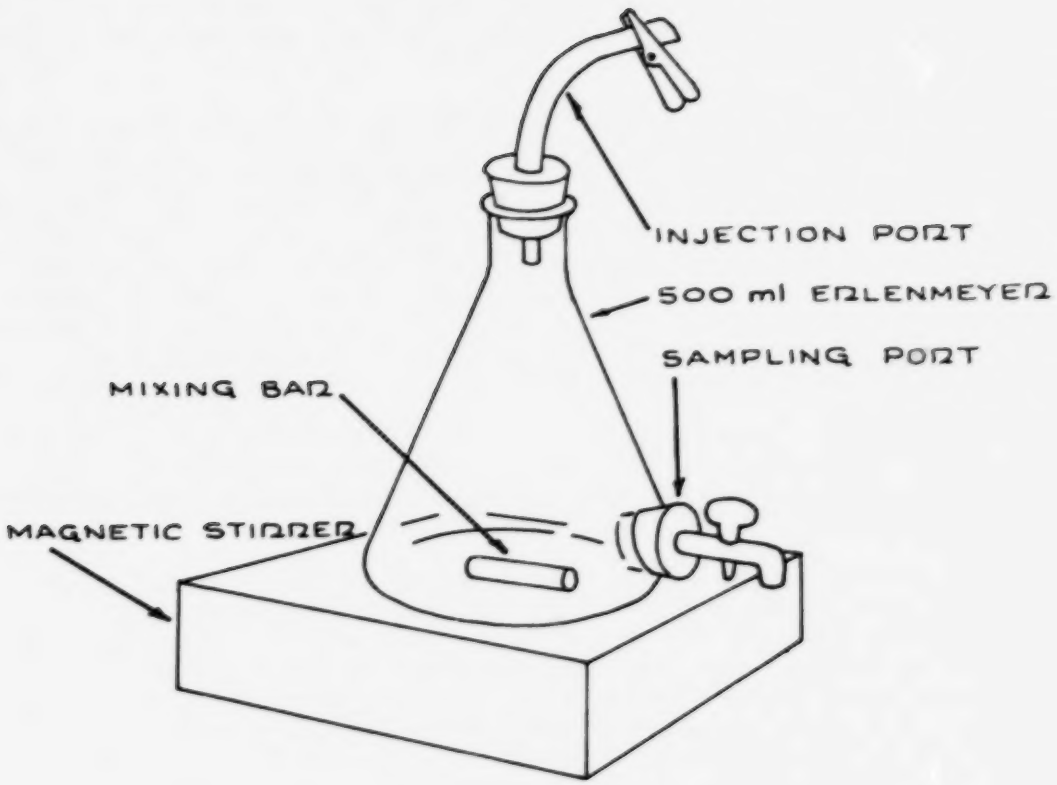

Figure 4. Batch Reactor With Declining Residual

flask with a bottom sidearm. A silicone stopper was used to attach a stopcock to the sidearm for use as a sampling outlet. A short length of tygon tube attached to a hollow glass rod was fitted to a single hole rubber stopper. The tube was closed with a clamp during the reaction pericd to prevent the ozone from escaping. Free flow of ozone at the sampling period was obtained by removing the clamp.

Following ozonation, 500 ml of TDW with 10^{-3} M phosphate buffer in NaCl (pH 7.0 $\pm$ 0.2) were transferred to the reactor. The reactor was placed on a magnetic stirrer (Fisher Scientific, Fair Lawn, N.J.) for the duration of the experiment. Triplicate aliquots of the ozonated, buffered TDW were drawn to determine the initial ozone residual in the reactor. The cell-associated virus sample was injected into the reactor from a 10 ml syringe. The outlet was purged 15 seconds before the designated contact time to flush possible residual virus from the stopcock at the sampling port. At specified contact times, samples were collected in ozone demand free test tubes. A 5.0 ml aliquot was used to determine ozone residual as previously described, while 3.0 ml were immediately mixed with 1.0 ml $Na_2S_2O_3$ utilizing a Vortex Genie (Fisher Scientific, Fair Lawn, N.J.). Surviving viruses were titrated and expressed in pfu/ml of reactor volume.

Batch Reactor for Continuous Ozonation

The ozonation apparatus as previously described (Figure 1) was used and the 20 L Pyrex dilution water bottle was replaced with a 2 L Pyrex reagent bottle (see Figure 5). An outlet cut in the reagent bottle was fitted with a silicone stopper with a stopcock. Injection of the sample was made with a 10 ml syringe through the stopper at the sampling port. Injection of the sample directly under the fritted glass immersion tube mixed the sample.

A 1500 ml volume of TDW with 10^{-3} phosphate buffer in NaCl (pH 7.0 $\pm$ .2) was ozonated to obtain a maximal ozone dosage. Following removal of triplicate samples for measurement of the initial ozone concentration within the reactor, the cell-associated virus was injected from a 10 ml syringe into the reactor. The outflow stopcock was purged 10 to 15 seconds before withdrawing samples at specified contact times. The samples were collected in ozone demand free test tubes with a 5 ml aliquot to determine ozone residual and a 3 ml aliquot, neutralized with 1 ml of $Na_2S_2O_3$, to determine the surviving viral titer.

Ozone Determination

Ozone concentrations were measured according to the method of Shechter (33) with minor modifications. Two calibration graphs were generated to measure ozone. Curve A was utilized to measure ozone concentrations ranging from 0.01 to 0.3 mg/L and Curve B was used to measure ozone ranging from 0.3 to 2.0 mg/L. In preparing the calibration graph for Curve A, the reference blanks were made with TDW and neutral potassium iodide reagent. Equal aliquots of the neutral potassium iodide reagent were placed in the reference blank and the standard with which it was to be compared. This served to zero out absorbance due to the potassium iodide. This modification was not necessary with the B curve. Low levels of ozone (curve A) were determined using a

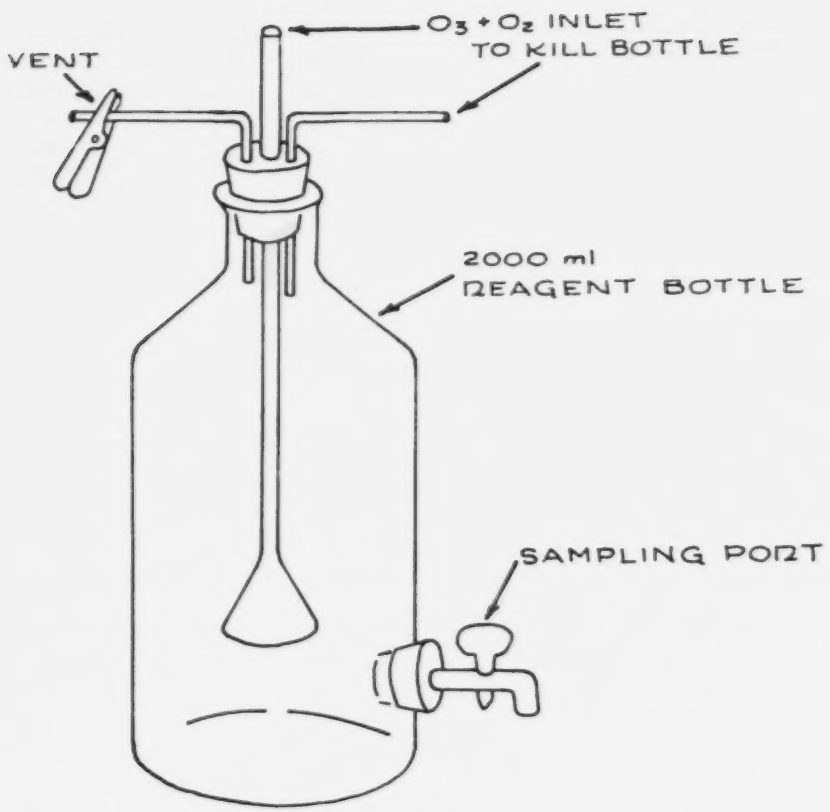

Figure 5. Batch reactor for continuous ozonation.

blank of 2 ml neutral potassium iodide reagent in 10 ml TDW. Absorbances were read in a Hitachi Perkin-Elmer Model 139 UV-Vis Spectrophotometer with a Model 139-0251 photomultiplier unit (Coleman Instruments Corporation, Maywood, Illinois). Curve A was prepared using Hellma 2 cm quartz cells (Hellma Cells, Jamaica, New York). Fisher brand 1 cm quartz cells (Fisher Scientific Company, Medford, Massachusetts) were used for curve B.

The ozone demand of the unadsorbed samples from the Sharp apparatus was found by comparing a clear buffer sample taken at 40 seconds and the 40 second unadsorbed sample. The demand of the fecal material was analyzed in a somewhat different manner. After injection of the initial sample which was used for organism inactivation and ozone determination, another 5 ml sample of fecal material was injected into the apparatus and a 10 ml sample was withdrawn at the 40 second sample port (fecal blank). This sample was combined with 2 ml TDW and placed in the dark for 30 minutes along with the other samples taken for ozone determination. The absorbance of this sample was read using a TDW reference blank. Since both fecal samples withdrawn at 40 seconds contained partially oxidized fecal material, a comparison of the two samples (fecal and ozone vs fecal) enabled determination of absorbance due solely to the ozone present. The ozone concentration of the clear buffer sample was compared to the ozone concentration of the adsorbed sample to obtain ozone demand.

SECTION 4

RESULTS AND DISCUSSION

THE EFFECT OF FECAL MATERIAL ON OZONE DISINFECTION OF COLIFORM BACTERIA AND ENTERIC VIRUSES IN WATER.

RESULTS

The approximate concentrations of the various microorganisms within the fecal samples prepared as discussed in materials and methods are presented in Table 1. As will be seen later, there is an apparent discrepancy between the concentrations presented in the fecal samples and initial concentrations of fecal-associated organisms available for disinfection. This apparent discrepancy was caused by a dilution of $1:2.9 \times 10^4$ of the fecal samples, which occurred during sample preparation and subsequent disinfection.

After preparing the fecal suspensions, an analysis was conducted to determine the particle size distribution. One hundred random particles were measured using a microscope. A stage micrometer was used to calibrate the microscope. These data are presented in Table 2. The size of fecal particles presented after preparation of fecal suspensions for disinfection experiments ranged from 1 to 18 μm in size. The particle size distribution is significant in that it approximates that which might be found after direct filtration of drinking water. Also, particles present in wastewater effluent after activated sludge treatment are within this size range.

The particle size distribution in finished water from three different direct filters (dualmedia, multimedia, and anthracite and garnet) were found by Tate et al (34) to be virtually identical. A 99 percent removal of 2.5 to 150 μm particles was accomplished in all three cases while all particles greater than 10 μm were removed. Effluent samples were collected prior to disinfection and analyzed to determine the solids distribution. Rickert and Hunter (35) found that the soluble fraction (<1 mμ) contained 93 percent of the total solids in an activated sludge plant effluent. One percent of the total solids were colloidal (1 mμ to 1 μm), a 5 percent supra-colloidal (1 to 100 μm) and 1 percent settleable (<100 μm). Thus, it is evident that the fecal particle size range used in this research is within the range one might expect to find in a field situation.

Viruses

Results of the inactivation of poliovirus (Sabin Type 1) and fecal-associated poliovirus at a turbidity of 5 NTU are presented in Table 3.

TABLE 1. CONCENTRATION OF MICROORGANISMS PER GRAM OF FECES (WET WEIGHT)

Microorganism	Concentration/gm
Poliovirus	5.2×10^5 PFU (7)[a]
Porcine Picornavirus	1.8×10^6 $TCID_{50}$ (2)
Coliform bacteria	2.9×10^5 MPN (10)

[a]Number of determinations

TABLE 2. FECAL PARTICLE SIZE DISTRIBUTION

Fecal Suspension	Particle Distribution (%) Particle Size (μm)						
	1	2	4	5	7	9	18
Infant	14	52	25	5	4	0	0
Porcine	13	56	21	6	3	0	1
Adult	0	56	30	12	1	1	0

TABLE 3. INACTIVATION OF POLIOVIRUS (SABIN TYPE 1) AND FECAL ASSOCIATED POLIOVIRUS (SABIN TYPE 1)

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Ozone[a] Residual (mg/L)	Initial Concentration (PFU/ml ozonated stream)	Inactivation (%) Time (seconds) 10	20	30
Unadsorbed[b] Poliovirus	0.085	0.067	2.4×10^3	100	100	100
	0.012	0.008	1.5×10^4	99.7	99.95	99.98
	0.012	0.004	5.1×10^2	100	100	100
Fecal Associated Poliovirus	0.024	-	0.2×10^0	100	100	100
	0.019	0.005	2.0×10^0	100	100	100
	0.013	-	1.3×10^1	0	100	45

[a]At 40 seconds

[b]Data from Table 10

Protection by the fecal material was demonstrated at an ozone concentration of 0.013 mg/L. At this ozone concentration, the survival of fecal-associated poliovirus with an initial concentration of 1.3×10^1 PFU/ml was noted at 10 and 30 seconds of reaction time. On the other hand, unadsorbed poliovirus with a titer 2.7 $\log_{10}$ PFU/mL higher than that of fecal-associated poliovirus showed complete inactivation within 10 seconds at an ozone concentration of 0.012 mg/L.

Further work with poliovirus was discontinued due to inactivation of poliovirus within the stored frozen fecal material. As shown in Table 4, titer of fecal associated poliovirus ranged from 1.4×10^4 to 1.3×10^6 PFU/gm feces after 12 days at -20°C. After 71 days, however, complete inactivation of all poliovirus within the samples had occurred.

The storage of poliovirus at -20°C should not have resulted in the marked decrease in titer described above. The delay which occurred during shipment of the samples, however, allowed sufficient time for possible generation of some viricidal agent and initiation of viral inactivation. Although the agent responsible for inactivation was not isolated, a compound such as ammonia may have been responsible. Ammonia is known to be a potent viricide (36). The generation of ammonia from urea associated with the samples is conceivable since the fecal samples were obtained from infants and may have been in contact with urine, a source of large quantities of urea.

TABLE 4. INACTIVATION OF FECAL-ASSOCIATED POLIOVIRUS (SABIN TYPE 1) AT -20°C

	Virus Concentration (PFU/gm fecal material) Number of Days Stored at -20°C	
Sample	12	71
1	2.0×10^5	0
2	4.0×10^5	0
3	1.4×10^4	0
4	1.1×10^6	
5	1.1×10^5	0[a]
6	1.3×10^6	

[a]Samples 4, 5, and 6 were combined for use in experiments.

Porcine picornavirus Type 3 (Strain ECPO-6) was also protected by adsorption to or encasement in fecal material. Data in Table 5 show that at an initial ozone concentration of 0.024 mg/L, 100 percent inactivation of 1.1 x 10^1 $TCID_{50}$/ml unadsorbed porcine picornavirus was obtained while survival of fecal associated porcine picornavirus at an initial concentration of 4.1 x 10^1 $TCID_{50}$/ml was demonstrated after 30 seconds of contact. Complete inactivation of 1.6 x 10^4 $TCID_{50}$/ml was accomplished at an ozone concentration of 0.22 mg/L.

A comparison of the data on the inactivation of unadsorbed poliovirus and the unadsorbed porcine picornavirus shows that the porcine picornavirus was significantly more resistant than was poliovirus. At a level of 0.12 mg/l ozone (Table 3) a four log reduction of poliovirus was accomplished. Porcine picornavirus, however, was reduced by only two logs when the ozone concentration was 0.036 mg/L

Some degree of caution should be exercised in viewing the data on the inactivation of poliovirus and porcine picornavirus since only one run demonstrating protection was obtained for each virus. In both instances, however, survival of fecal-associated virus was noted after 30 seconds contact whereas total inactivation of non-associated viruses was accomplished within 10 seconds.

Bacteria

Washed cultures of E. coli were subjected to ozone levels ranging from 0.38 mg/L to 0.62 mg/L. Samples were removed at contact times of 10, 20, 30, and 40 seconds and assayed for survival. These data are presented in Table 6. For a given contact time the inactivation does not appear to vary significantly over the range of ozone levels used. However, the results do vary with contact time. An average of 5.0, 5.8, 6.8, and 9.4 logs of reduction were observed at 10, 20, 30, and 40 seconds, respectively. The average ozone concentration utilized to accomplish these reductions was 0.52 mg/L.

Table 6 also contains the results of the inactivation of S. fecalis. While a limited amount of work was conducted with this organism, it is evident that S. fecalis was more sensitive to ozone than was E. coli. Complete inactivation of S. fecalis within 10 seconds was accomplished at initial ozone concentrations of 0.28 mg/L and above. At an ozone concentration of 0.13 mg/L survival was noted at 10, 20, and 30 seconds, although the level of bacteria had been reduced by 7 logs within 30 seconds of ozonation.

Data obtained on the inactivation of E. coli and fecal associated coliforms are presented in Table 7. The samples which were assayed for survival were diluted tenfold to yield 1/10 and 1/100 dilutions. One ml aliquots of the diluted samples were then placed in tubes of lactose broth. In addition, the remaining undiluted sample was distributed equally between 5 tubes of lactose broth. Aliquots of undiluted samples normally ranged from 0.35 to 0.50 ml. Since one ml aliquots were not available for MPN determinations of surviving organisms, all MPN indices were reported as the actual values for the amount of sample used.

TABLE 5. INACTIVATION OF PORCINE PICORNAVIRUS TYPE 3 (STRAIN ECPO-6) AND FECAL-ASSOCIATED PORCINE PICORNAVIRUS TYPE 3 (STRAIN ECPO-6)

pH = 7.0 ± 0.2 Temperature

	Initial Ozone (mg/L)	Ozone[a] Residual (mg/L)	Initial Concentration ($TCID_{50}$/ml ozonated stream)	Inactivation (%) Time (seconds) 10	20	30
Unadsorbed Porcine Picornavirus	0.220	0.051	1.6×10^4	100	100	100
	0.037	0.016	8.7×10^4	33	=	=
	0.036	0.018	2.3×10^4	=[b]	97	98
	0.024	0.017	1.1×10^1	100	100	100
Fecal Associated Porcine Picornavirus at NTU = 5	0.037	0.016	1.1×10^1	100	100	100
	0.026	-	1.1×10^0	100	100	100
	0.024	-	4.1×10^1	0	83	76

[a]At 40 seconds

[b]Survival noted, however the titer could not be calculated

TABLE 6. INACTIVATION OF ESCHERICHIA COLI AND STREPTOCOCCUS FECALIS

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

Initial Ozone (mg/L)	Initial Concetration (CFU/100 ml ozonated stream)	Inactivation (%) Time (seconds) 10	20	30	40
		Escherichia Coli			
0.62	4.9×10^9	99.9996	99.99990	99.9993	-
0.57	7.9×10^9	-	99.999996	100	100
0.57	1.6×10^{10}	99.99995	99.99997	100	-
0.57	3.6×10^9	99.992	99.9990	100	-
0.56	4.0×10^9	99.997	99.996	99.9998	-
0.55	1.4×10^9	99.9990	99.9997	99.99998	-
0.54[a]	1.1×10^9	-	99.9996	99.99994	100
0.52[b]	3.1×10^9	99.9991	-	99.9995	-
0.51	2.4×10^9	-	99.999990	-	100
0.48	1.8×10^9	99.9997	99.9990	99.99998	-
0.41	6.4×10^{10}	99.995	99.998	99.9991	-
0.38	1.1×10^{10}	99.9991	99.99997	99.9998	-
		Streptococcus Fecalis			
		10	20	30	
0.54[a]	2.6×10^8	100	-	-	
0.52[b]	2.3×10^8	100	-	100	
0.45	5.6×10^8	100	100	-	
0.28[a]	1.5×10^9	100	-	100	
0.13	1.5×10^8	99.99990	-	99.999990	
0.13	3.9×10^8	99.9998	99.9998	-	

a temp = 23°C b pH = 6.4

TABLE 7. INACTIVATION OF ESCHERICHIA COLI AND FECAL-ASSOCIATED COLIFORMS

pH = 7.0 ± 0.2 temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Ozone Residual (mg/L)	Initial Concentration (MPN/100 ml ozonated stream)	Inactivation (%) Time (seconds) 10	20	30
Unadsorbed *E. coli*	0.102	-	3.4×10^3	100[b]	100	100
	0.096	0.067	1.1×10^6	100	100	100
	0.066	0.055	8.3×10^3	100	100	100
	0.036	0.011	6.9×10^2	100	100	100
	0.028	-	5.9×10^3	100	100	100
	0.010	-	6.9×10^1	100	100	100
Fecal Associated Coliforms at NTU = 1	0.037	0.004	9.3×10^1	100	100	100
	0.028	-	1.1×10^2	100	100	100
Fecal Associated Coliforms at NTU = 5	0.620	-	1.1×10^4	100	100	100
	0.096	-	4.5×10^3	97	100	100
	0.066	-	1.7×10^2	100	100	100
	0.037	0.007	3.2×10^3	62	100	100
	0.028	-	9.7×10^2	62	75	100
	0.028	-	4.5×10^3	89	85	94
	0.027	0.014	1.7×10^2	81	0	24
	0.010	-	4.8×10^3	62	77	32
	0.010	-	1.9×10^4	60	87	82

a At 40 seconds b 100 percent means all MPN tubes inoculated were negative

Complete inactivation of non-associated E. coli occurred at all levels of ozone. Protection of fecal-associated coliform bacteria at a NTU of 5 was demonstrated at 0.10 mg/L and below. A definite gradient of survival was established during disinfection of fecal-associated coliforms since increased levels of survival were obtained with decreasing levels of ozone. It should be noted that the initial MPN's of the unadsorbed washed E. coli were generally higher than those of the corresponding fecal-associated samples. Even though the initial MPN's of the washed culture's were greater, the E. coli were completely inactivated within 10 seconds while contact times of 10 to 30 seconds or more were necessary to accomplish total inactivation of fecal-associated coliform bacteria. Thus, it is evident that the rate of inactivation of non-associated E. coli was more rapid than was the rate of inactivation of fecal-associated coliforms.

No protection of fecal-associated coliform bacteria was demonstrated at 1 NTU. This may have been due in part to the lower levels of bacteria present. Total inactivation of 1.1×10^2 MPN/100 ml at 1 NTU occurred within 10 seconds at an ozone concentration of 0.028 mg/L. Unadsorbed E. coli at an initial concentration of 5.9×10^3 MPN/100 ml were also completely inactivated within 10 seconds at the same ozone concentration. Since total inactivation of unadsorbed E. coli and fecal-associated coliforms at 1 NTU occurred within 10 seconds, the rate of inactivation of the organisms could not be determined. Thus, whether or not 1 NTU of fecal material afforded protection to fecal-associated coliforms could not be ascertained. In comparison, survival was evident at 5 NTU after subjecting 1.7×10^2 MPN/100 ml to 0.027 mg/L ozone for 30 seconds. In fact, a log reduction of only 0.8 was accomplished during this contact period. It would appear that the greater amount of organic material at 5 NTU did offer protection of fecal-associated coliforms while little or no protection was afforded at 1 NTU.

An experiment was conducted to determine whether those coliforms surviving ozonation did so because of the protective effect of the fecal material or because they were resistant forms. E. coli which survived ozonation when associated with fecal material (ECF) were isolated as described in Materials and Methods. Comparisons were then made of the inactivation rates of washed cultures of E. coli (ATCC 15766) and washed cultures of isolated E. coli. This procedure was repeated twice with a separate E. coli isolate used in each trial. These data may be seen in Table 8. No significant differences were noted between the two organisms. In the first trial both E. coli and ECF were reduced by slightly more than 5 logs within a 30 second contact period. In the second trial E. coli and ECF underwent reductions of approximately 6 and 5 logs, respectively. Therefore, it would appear that survival of fecal-associated coliform bacteria occurs as a result of protection afforded the bacteria by the fecal material rather than some inherent resistance of the organisms themselves.

DISCUSSION

The amount of work conducted with the poliovirus and the porcine picornavirus was limited because of several problems which occurred during the course of investigation. Studies with both poliovirus and porcine picornavirus were

TABLE 8. INACTIVATION OF *E. COLI* (ATCC 15766) AND *E. COLI* SURVIVING OZONATION WHEN ASSOCIATED WITH FECAL MATERIAL

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Ozone[a] Residual (mg/L)	Initial Concentration (MPN/100 ml ozonated stream)	Inactivation (%) Time (seconds) 10	20	30
E. coli	0.58	0.27	2.4×10^7	99.994	99.994	99.9992
ECF[b]	0.58	0.21	5.9×10^7	99.994	99.9996	99.9994
E. coli	0.56	-	1.9×10^8	99.9990	99.9998	99.9998
ECF	0.56	0.22	1.7×10^7	99.98	99.995	99.998

[a] At 40 seconds

[b] ECF = *E. coli* which survived ozonation when associated with fecal material

hindered by the low levels of viruses available for disinfection after sample preparation. There was also difficulty in obtaining fecal samples which contained naturally occurring viruses, i.e. samples containing viruses which been excreted in the feces. Difficulty in obtaining sufficient quantities of these samples and the inactivation of poliovirus within fecal samples stored at -20°C abruptly curtailed work with this organism.

Despite the problem in working with low levels of viruses, these levels closely approximate the levels of organisms which might normally go through disinfection in a wastewater treatment plant. When one considers that levels of viruses as low as 1 $TCID_{50}$ or 2 PFU are capable of producing infection in man under certain conditions (12), low levels of viruses should be considered significant.

The level of viruses present in domestic wastewater has been estimated to be about 7000 PFU/L (10). Assuming an influent level of 7000 PFU/L, Sproul (12) predicted the number of viruses that would remain after wastewater treatment. Utilizing these data, the number of viruses which would be subject to disinfection during secondary treatment may be determined. The level of viruses likely to remain after trickling filtration would be approximately 3500 PFU/L and about 700 PFU/L would be expected after treatment with activated sludge. These figures correlate well with the range of fecal-associated poliovirus (2.0×10^2 to 1.3×10^4 PFU/L) which were employed during these disinfection experiments. Fecal-associated porcine picornavirus ranged from 1.1×10^3 to 4.1×10^4 $TCID_{50}$/L.

Samples of fecal-associated coliforms were similar to fecal-associated enteric viruses in that low levels of organisms were obtained after cleanup of the fecal samples. In order to increase the concentration of bacteria, the fecal solution was centrifuged at 7500 x g during the washing procedure rather than at the lower centrifgual force. Since more material was pelleted at this higher centrifugal force, the levels of fecal-associated coliforms and the turbidity of the sample were increased. It should be noted, however, that centrifugation at 7500 x g resulted in removal of non-associated, as well as associated coliforms from the suspension. Despite this fact, protection of fecal-associated coliforms was seen. A few of those coliforms surviving ozonation may not have been adsorbed to or encased in fecal material.

The small ozone residual remaining after disinfection was difficult to measure spectrophotometrically. Detection was further complicated by the large quantity of partially oxidized fecal material present in the sample. To alleviate this problem a blank containing partially oxidized fecal material was used to zero out absorbance due to the fecal material. This procedure was not always effective, however, due to the large amounts of absorbance which had to be zeroed out.

It is uncertain whether the protection afforded the microorganisms by the fecal material was due to complete depletion of the initial ozone in solution or adsorption of the microorganisms to or encasement in fecal material. The increase in inactivation with longer contact times, however, seems to indicate that complete depletion of ozone did not occur within the 30 second contact period.

Although the results of this investigation demonstrated protection of fecal-associated coliform bacteria and enteric viruses, the significance of this protection is small when viewed with respect to common ozone disinfection practices. In practice, ozone residuals of 0.1 to 0.2 mg/L and 0.4 mg/L at the end of a contact time of about 4 minutes were maintained to disinfect bacteria and viruses, respectively. Diaper (37) reported that an applied dosage of 0.5 to 1.5 mg/L ozone in drinking water was necessary to obtain these residuals which are determined after a 5 minute contact period. Other waters might require a larger applied dosage. In comparison, the initial ozone in solution in these studies ranged from 0.010 to 0.22 mg/L with a few exceptions and the residual ozone, measured after 40 seconds contact, ranged from 0.005 to 0.067 mg/L. The ozone concentrations necessary to inactivate fecal-associated organisms were somewhat higher than those required fecal-associated organisms. However, at some point within the range of ozone levels employed, it was possible to achieve 100 percent inactivation of both fecal-associated and non-associated organisms within 30 seconds.

THE EFFECT OF HYDRATED ALUMINUM OXIDE FLOC ON OZONE DISINFECTION OF *E. COLI* AND VIRUSES IN WATER.

RESULTS

The range of floc particle sizes used for this experimentation from light microscope observations was from 5 to 50 μm. Although a portion of the particles used for O_3 disinfection studies were on the high side of the 10 μm particle size cited, use of this fraction probably provided a factor of safety since if no protection was evident using these slightly larger particles, it is probable that no protection would be provided by the fraction less than 10 μm.

Poliovirus

As shown in Table 9, after adsorption and subsequent centrifugation of the adsorbed poliovirus 1, the analysis of the supernatant indicated that approximately 1 percent of the initial concentration introduced into the adsorption reactor was not associated with the floc. Therefore, by computation, 99 percent of the initial titer was associated with the floc. Titratation of the floc, which indicates the viruses actually recoverable from the adsorbed fraction, yielded a recovery of 114 percent. Titration of the supernatant, after homogenization and centrifugation, indicated that approximately 2 percent of the adsorbed virus was released by homogenization. It is evident, therefore, that the virus particles were held tightly by the floc, and mechanical shear did not release them. Titration of the floc following homogenization showed a 121 percent recovery of the adsorbed fraction. Virus losses due to the adsorption and centrifugation process in the TDW control were only about 1 percent.

The O_3 concentration range investigated for poliovirus disinfection, shown in Table 10 was from 0.002 to 0.0335 mg/l. The data indicate the the poliovirus was afforded no protection by either 1 or 5 NTU of hydrated aluminum oxide floc at the concentration levels of O_3 utilized. At an initial

TABLE 9. ASSOCIATION OF POLIOVIRUS (SABIN TYPE 1) WITH HYDRATED ALUMINUM OXIDE

SYSTEM	RANGE OF VIRUS CONCENTRATIONS[a] (PFU/ml)	VIRUS RECOVERY (%)
Computed virus concentration in floc $\frac{(\text{initial-supernatant})}{\text{initial}} \times 100$	2.5×10^1 to 3.5×10^3	99.0 (5)[b]
Actual recovery from floc (adsorbed) $\frac{\text{adsorbed}}{\text{computed}} \times 100$	3.2×10^4 to 1.9×10^5	114 (5)
Computed virus concentration in floc after homogenization $\frac{(\text{adsorbed-homogenized supernatant})}{\text{adsorbed}} \times 100$	1.5×10^2 to 3.3×10^3	98 (5)
Actual recovery from floc after homogenization $\frac{(\text{homogenized-homogenized supernatant})}{(\text{adsorbed-homogenized supernatant})} \times 100$	8.0×10^4 to 1.7×10^5	121 (4)
TDW control $\frac{\text{control}}{\text{initial}} \times 100$	1.1×10^5 to 1.3×10^5	99.3 (2)

[a] range of initial concentration: 2.9×10^4 to 1.7×10^5

[b] number of trials

TABLE 10. INACTIVATION OF POLIOVIRUS (SABIN TYPE 1) UNADSROBED AND ADSORBED AT 1 AND 5 TURBIDITY UNITS (NTU) HYDRATED ALUMINUM OXIDE FLOC

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Residual[a] Ozone (mg/L)	Initial Concentration (PFU/ml ozonated stream)	Inactivation (Percent) Time (seconds) 10	20	30
UNADSORBED	0.085	0.067	2.4×10^3	100	100	100
	0.018	0.017	8.4×10^3	99.96	100	100
	0.015	0.011	1.4×10^4	99.6	99.98	99.98
	0.012	0.008	1.5×10^4	99.7	99.95	99.98
	0.012	0.004	5.1×10^2	100	100	100
	0.006	-	8.3×10^3	99.8	100	99.96
1 NTU	0.085	-	1.8×10^3	100	100	100
ADSORBED	0.018	0.008	7.7×10^3	99.3	99.91	99.91
	0.015	0.009	7.9×10^3	100	100	100
	0.012	0.002	1.1×10^3	100	100	100
	0.006	-	5.6×10^3	100	99.94	99.94
	0.002	-	7.6×10^3	99.6	99.7	99.96
5 NTU	0.012	-	1.2×10^4	99.97	99.97	100
ADSORBED	0.012	-	1.0×10^4	99.93	100	100

[a] taken at 40 seconds

O_3 concentration of 0.012 mg/L, 99.95 percent of unadsorbed poliovirus with a concentration of 1.5×10^4 PFU/ml were inactivated after 20 seconds of O_3 contact. At the same O_3 concentration 99.97 and 100 percent of floc-associated poliovirus at 5 NTU with concentrations of 1.2×10^4 and 1.0×10^4 PFU/ml, respectively, were inactivated within 20 seconds of contact. At 1 NTU of hydrated aluminum oxide floc at an initial O_3 concentration of 0.012 mg/L complete inactivation of 1.1×10^3 PFU/ml floc associated poliovirus was also accomplished within 20 seconds. At 0.085 mg/L O_3 complete inactivation of 3 logs of both unadsorbed and adsorbed poliovirus at 1 NTU was achieved within 10 seconds.

Coxsackievirus A9

Data in Table 11 show that, based on the supernatant titer obtained following centrifugation, the adsorption of coxsackievirus A9 was approximately 99 percent, as was the case for the poliovirus 1. Titration of the floc showed a recovery of 96 percent of the adsorbed virus. The virus released by homogenization again paralleled the poliovirus, with only 2 percent of the adsorbed fraction released. An assay of the adsorbed sample after homogenization yielded a recovery of approximately 100 percent indicating no losses due to homogenization. The TDW control indicated a coxsackievirus recovery of 94 percent following the adsorption procedure and subsequent centrifugation step.

As shown in Table 12, the disinfection data for the coxsackievirus also paralleled those of the poliovirus very closely. The O_3 concentration range was from 0.012 to 0.081 mg/L. Again, the data did not seem to indicate a protective effect at either 1 or 5 NTU over the concentration range investigated. At an initial O_3 concentration of 0.032 mg/L, 99.8 and 99.0 percent of unadsorbed coxsackievirus with initial concentrations of 2.2×10^4 and 9.1×10^3 PFU/ml, respectively, were inactivated after 10 seconds of O_3 contact. Using the same O_3 concentration and contact time, 99.8 percent of coxsackievirus with an initial concentration of 2.4×10^4 PFU/ml was inactivated when associated with 1 NTU of hydrated aluminum oxide floc. Also, at 5 NTU and 0.032 mg/L, O_3 99.90 percent of floc-associated coxsackievirus with a titer of 9.1×10^3 PFU/ml was inactivated after 10 seconds of contact. At an initial O_3 concentration of 0.081 mg/L, complete inactivation of 4 logs/ml of coxsackievirus both unadsorbed and adsorbed at 1 NTU of hydrated aluminum oxide floc was accomplished within 10 seconds.

The disinfection of poliovirus and coxsackievirus appeared to be very similar in that they were both very sensitive to the O_3 concentrations used. For both viruses the O_3 concentration range investigated was less than 0.1 mg/L. No protection at either 1 or 5 NTU of hydrated aluminum oxide was evident for either virus over the very similar O_3 concentration range used.

Bacteriophage f_2

The f_2 bacteriophage data in Table 13 show that the adsorption, computed using the supernatant titer obtained following centrifugation, was 90 percent of the initial virus introduced into the adsorption reactor. Titration of

TABLE 11. ASSOCIATION OF COXSACKIEVIRUS A9 WITH HYDRATED ALUMINUM OXIDE

SYSTEM	RANGE OF VIRUS CONCENTRATIONS[a] (PFU/ml)	VIRUS RECOVERY (%)
Computed virus concentration in floc $\frac{(\text{initial-supernatant})}{\text{initial}} \times 100$	2.0×10^2 to 6.2×10^3	99.0 (6) [b]
Actual recovery from floc (adsorbed) $\frac{\text{adsorbed}}{\text{computed}} \times 100$	1.1×10^5 to 3.4×10^5	96 (6)
Computed virus concentration in floc after homogenization $\frac{(\text{adsorbed-homogenized supernatant})}{\text{adsorbed}} \times 100$	1.6×10^3 to 5.3×10^3	98 (5)
Actual recovery from floc after homogenization $\frac{(\text{homogenized-homogenized supernatant})}{(\text{adsorbed-homogenized supernatant})} \times 100$	1.3×10^5 to 3.1×10^5	99.98(5)
TDW control $\frac{\text{control}}{\text{initial}} \times 100$	-	94 (3)

[a] Range of initial concentration: 1.4×10^5 to 3.2×10^5

[b] Number of trials

TABLE 12. INACTIVATION OF COXSACKIEVIRUS A9 UNADSORBED AND ADSORBED AT 1 AND 5 NTU OF HYDRATED ALUMINUM OXIDE FLOC

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Residual[a] Ozone (mg/L)	Initial Concentration (PFU/ml ozonated stream)	Inactivation (%) Time (seconds)		
				10	20	30
UNADSORBED	0.081	0.042	1.3×10^4	100	100	100
	0.051	0.023	1.5×10^4	99.98	100	100
	0.032	-	2.2×10^4	99.8	99.98	100
	0.032	0.027	9.1×10^3	99.0	99.7	99.90
	0.015	0.012	1.3×10^4	96	99.0	99.0
	0.012	-	1.2×10^4	90	92	96
	0.012	0.010	9.1×10^3	97	99.2	99.8
1 NTU ADSORBED	0.081	0.019	1.0×10^4	100	100	100
	0.051	0.012	1.4×10^4	99.8	100	100
	0.032	0.013	2.4×10^4	99.8	100	100
	0.012	-	8.4×10^3	95	97	98
5 NTU ADSORBED	0.032	-	9.1×10^3	99.90	100	100
	0.015	-	1.5×10^4	99.1	99.5	99.98
	0.012	-	9.8×10^3	99.0	99.93	100

[a]Taken at 40 seconds

TABLE 13. ASSOCIATION OF f_2 BACTERIOPHAGE WITH HYDRATED ALUMINUM OXIDE

SYSTEM	RANGE OF VIRUS CONCENTRATIONS[a] (PFU/ml)	VIRUS RECOVERY (%)	
Computed virus concentration in floc $\frac{(\text{initial-supernatant})}{\text{initial}} \times 100$	5.0×10^4 to 1.6×10^7	90	(5)[b]
Actual recovery from floc (adsorbed) $\frac{\text{adsorbed}}{\text{computed}} \times 100$	1.6×10^4 to 8.9×10^4	0.04	(4)
Computed virus concentration in floc after homogenization $\frac{(\text{adsorbed-homogenized supernatant})}{\text{adsorbed}} \times 100$	5.0×10^2 to 2.0×10^4	96	(3)
Actual recovery from floc after homogenization $\frac{(\text{homogenized-homogenized supernatant})}{(\text{adsorbed-homogenized supernatant})} \times 100$	2.1×10^4 to 1.2×10^5	93	(3)
TDW control $\frac{\text{control}}{\text{initial}} \times 100$	1.5×10^7 to 4.6×10^7	19	(4)

[a]Range of initial concentration: 6.1×10^7 to 2.0×10^8

[b]Number of trials

the adsorbed fraction, however, indicated a recovery of less than 0.05 percent of the phage from the hydrated aluminum oxide floc. Thus, the overall untitratable adsorbed virus was approximately 99.95 percent. It was uncertain whether the inability to recover the adsorbed virus was due to inactivation or failure to elute the phage from the floc. Therefore, in an attempt to recover more of the virus, sonication was used at a level of sonic energy used by Boardman and Sproul (29) for minimum phage loss. No significant increase in recovery was observed. A loss of bacteriophage amounting to approximately 81 percent occurred during the adsorption procedure and subsequent centrifugation, as indicated by the recovery of virus from the TDW control. The supernatant titer obtained after centrifugation of the homogenized floc indicated a 4 percent release of phage during this process. Titration of the floc following homogenization showed a recovery of 93 percent of the adsorbed fraction, thus indicating that 7 percent of the adsorbed phage was unrecoverable after homogenization.

Table 14 contains the compiled data for the inactivation of f_2 bacteriophage over a range of O_3 concentrations from 0.115 to 2.18 mg/L. Caution must be exhibited in interpreting these data since there is the possibility that some phage was still incorporated within the floc particles and was unrecoverable. Therefore, the initial titer for the adsorbed phage is given in two forms in Table 14. The first represents the initial titer computed assuming viable virus was present in the floc but unrecoverable, while the second represents the virus what was actually recovered (as previously indicated, less than 0.05 percent minus the losses incurred by homogenization). If the computed initial titers of adsorbed f_2 bacteriophage are used, then no protection was evident at either 1 or 5 NTU. If the actual titers of recoverable viruses were used, a partial protective effect may be afforded. At an initial O_3 concentration of 0.127 mg/L unadsorbed bacteriophage with an initial concentration of 8.4×10^6 PFU/ml was reduced 5.9 logs within 30 seconds. At the same O_3 concentration using the initial computed titer of 7.7×10^6 PFU/ml, a 6.7 log reduction was obtained after 30 seconds of O_3 contact when the bacteriophage was associated with 1 NTU of hydrated aluminum oxide floc. When the actual titer of recoverable phage was used, a 3.0 log reduction of f2 with an initial concentration of 1.5×10^3 PFU/ml was obtained after 30 seconds of O3 contact. However, the data at both 1 and 5 NTU are inconclusive with respect to protection, since the actual extent of inactivation of the adsorbed phage could not be determined due to the low levels of recovery from the floc.

Escherichia coli

As previously indicated in the sample preparation section of Materials and Methods, the adsorbed E. coli samples were not centrifuged to separate the unadsorbed from the adsorbed bacteria. Instead settling analyses done on a portion of both the adsorbed and homogenized samples were used to calculate the unadsorbed fraction and the fraction released by homogenization. As indicated, an E. coli control was also established and analyzed to determine the natural inactivation and sedimentation of bacteria within 24 hours. The results of 7 trials showed a 69 percent recovery of E. coli in 24 hours at 4°C. Six percent of this recovered fraction had settled in the 24 hour time span. Correction factors derived from these results were applied to the

TABLE 14. INACTIVATION OF f_2 BACTERIOPHAGE UNADSORBED AND ADSORBED AT 1 AND 5 NTU OF HYDRATED ALUMINUM OXIDE FLOC

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Residual[a] Ozone (mg/L)	Initial Concentration (PFU/ml ozonated stream)	Inactivation (%) Time (seconds) 10	20	30
UNADSORBED	2.18	-	3.4×10^6	100	100	100
	0.79	-	8.4×10^6	99.9992	99.9996	99.999992
	0.53	-	1.0×10^8	99.9996	99.99997	99.999995
	0.33	0.28	1.3×10^7	99.9994	99.99990	99.99998
	0.127	0.119	8.4×10^6	99.98	99.998	99.99990
	0.115	0.083	7.0×10^6	99.997	99.9997	99.9998
1 NTU ADSORBED	0.127	0.056	7.7×10^{6b}	99.99990	99.99993	99.99998
			1.5×10^{3c}	99.3	99.6	99.91
5 NTU ADSORBED	2.18	-	6.9×10^{6b}	100	100	100
			1.5×10^{3c}	100	100	100
	0.79	-	9.2×10^6	99.99995	99.99997	99.99998
			1.9×10^3	99.8	99.90	99.90
	0.33	-	1.6×10^7	99.99996	100	100
			8.4×10^3	99.92	100	100
	0.115	-	1.7×10^7	99.9998	99.99992	99.99998
			2.2×10^3	99.0	99.4	99.90

[a] taken at 40 seconds [b] theoretical titer [c] actual titer

supernatant values obtained from the 24 hour settling analyses on both the adsorbed and the homogenized samples to account for losses incurred by both natural inactivation and sedimentation. The computed E. coli adsorption onto the floc, as shown in Table 15, based on the average supernatant titer of 2 percent following sedimentation, was 98 percent of the initial bacteria introduced. Titration of the adsorbed fraction showed that 91 percent of the bacteria were recoverable from the hydrated aluminum oxide floc. The supernatant titer obtained after sedimentation of homogenized floc showed that 11 percent of the bacteria were released during this process. The computed concentration that remained associated with the floc was, therefore, 89 percent.

The E. coli dimensions (0.3 x 1-5 μm), as compared to the floc particles (5 to 50 μm), indicate the possibility that more than one bacterium may have been associated with each floc particle. In the assay technique employed for E. coli enumeration a floc particle, possibly containing multiple bacteria, may have produced only one colony when plated thereby indicating a source of bacteria loss.

After sample homogenization, 11 percent of the adsorbed E. coli may have been more available for release when the floc particles were sheared. Titration of the floc particles following homogenization showed that 100 percent of the adsorbed bacteria associated with the floc were recoverable. The TDW control in this case yielded 100 percent recovery of E. coli, indicating no losses due to the adsorption procedure.

The O_3 disinfection range investigated for E. coli, shown in Table 16, was from 0.014 to 0.53 mg/L of O_3. At an initial O_3 concentration of 0.021 mg/L 99.7 percent of unadsorbed E. coli with an initial concentration of 1.5×10^8 CFU/100 ml were inactivated after 10 seconds of O_3 contact. At the same O_3 concentration, 99.8 percent of floc-associated E. coli at 1 NTU with an initial concentration of 1.3×10^8 CFU/100 ml were also inactivated within 10 seconds. At an initial O_3 concentration of 0.040 mg/L, 4.7 log reduction of unadsorbed E. coli were inactivated within 30 seconds when the initial bacterial concentration was 7.7×10^7 CFU/100 ml. At the same O_3 concentration 5.0 log reduction associated with 5 NTU of hydrated aluminum oxide floc were also inactivated within 30 seconds when the initial concentration of adsorbed bacteria was 7.0×10^7 CFU/100 ml. An initial O_3 concentration of 0.239 mg/L resulted in complete inactivation of both unadsorbed and adsorbed E. coli at 5 NTU in 30 seconds with initial concentrations of approximately 8 logs/100 ml. The values cited above indicate that no protective effect was afforded the E. coli at either 1 or 5 NTU of hydrated aluminum oxide floc over the O_3 concentration range investigated (0.014 to 0.053 mg/L). It must also be remembered that only 0.1 ml aliquots of E. coli were plated. Therefore, on a colony/100 ml basis, a difference of only one colony became a difference of 1×10^3, possibly accounting for some of the variations in the data presented.

DISCUSSION

Hoff (17) has summarized recent virus studies involving disinfection

TABLE 15. ASSOCIATION OF *ESCHERICHIA COLI* WITH HYDRATED ALUMINUM OXIDE

SYSTEM	RANGE OF BACTERIAL CONCENTRATIONS[a] (CFU/100 ml)	BACTERIAL RECOVERY (%)
Computed bacterial concentration in floc $\frac{(\text{initial-supernatant})}{\text{initial}} \times 100$	1.2×10^7 to 1.1×10^8	98 (6)[b]
Actual recovery from floc (adsorbed) $\frac{(\text{adsorbed-supernatant})}{\text{computed}} \times 100$	8.7×10^8 to 2.3×10^9	91 (5)
Computed bacterial concentration in floc after homogenization $\frac{(\text{adsorbed-homogenized supernatant})}{(\text{adsorbed-supernatant})} \times 100$	2.6×10^7 to 2.9×10^8	89 (3)
Actual recovery from floc after homogenization $\frac{(\text{homogenized-homogenized supernatant})}{(\text{adsorbed-homogenized supernatant})} \times 100$	9.1×10^8 to 1.9×10^9	100 (3)
TDW control $\frac{\text{control}}{\text{initial}} \times 100$	1.4×10^9	100 (1)

[a]Range of initial concentration: 9.6×10^8 to 2.0×10^9

[b]Number of trials

TABLE 16. INACTIVATION OF ESCHERICHIA COLI UNADSORBED AND ADSORBED AT 1 AND 5 NTU OF HYDRATED ALUMINUM OXIDE FLOC

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Residual[a] Ozone (mg/L)	Initial Concentration (CFU/100 ml ozonated stream)	Inactivation (%) Time (seconds) 10	20	30
UNADSORBED	0.53	0.48	2.0×10^8	99.9998	100	100
	0.239	0.174	9.8×10^7	99.9990	99.9993	100
	0.040	0.029	7.7×10^7	99.8	99.96	99.998
	0.021	0.012	1.5×10^8	99.7	99.997	100
	0.018	0.005	1.4×10^8	97	99.94	99.98
	0.018	0.014	5.8×10^7	64	96	99.8
	0.018	-	5.1×10^7	99.98	100	99.990
	0.017	0.014	5.2×10^7	95	99.8	99.93
	0.014	-	1.0×10^8	86	94	99.2
1 NTU ADSORBED	0.021	0.006	1.3×10^8	99.8	99.996	100
	0.018	0.003	6.4×10^7	48	92	99.4
	0.018	0.008	7.7×10^7	99.990	99.998	99.9991
	0.017	0.004	5.5×10^7	93	99.3	99.6
5 NTU ADSORBED	0.239	-	1.3×10^8	100	100	100
	0.040	-	7.0×10^7	-	99.9990	99.9990
	0.018	-	1.1×10^8	98	-	99.992
	0.014	-	9.8×10^7	7	58	90

[a] taken at 40 seconds

with HOCl by stating that virus associated with inorganic particles such as clays or Al_3PO_4 showed inactivation rates similar to or perhaps slightly slower than freely suspended virus. This, compared to the disinfection rates of cell-associated viruses, indicated various degrees of protection, with some complexes exhibiting extreme resistance to disinfection. These findings, in conjunction with the results obtained from this study, strongly indicate that the lack of protection shown was directly related to the inorganic nature of the hydrated aluminum oxide floc. Chemical reactivity was probably minimal between the O_3 and the floc particle, and it would further appear that there was no physical interference provided by the floc. It is evident, since some of the bacteria and viruses were contained within the particles, that O_3 easily diffused into the hydrated aluminum oxide flocs in the size ranges used in this study and inactivated these organisms as readily as it did those attached to the surface of the floc. It can be concluded, therefore, that no major problem with ozone disinfection of the viruses and bacteria investigated would occur if these organisms were associated with alum floc particles of the size which might escape filtration of potable water supplies.

THE EFFECT OF HUMAN EPITHELIAL CARCINOMA CELLS ON OZONE DISINFECTION OF POLIOVIRUS (SABIN TYPE I) AND COXSACKIEVIRUS A9.

RESULTS

The inactivation data from the Sharp apparatus for the HEp-2 cell-associated poliovirus at 1 and 5 NTU are shown in Table 17. These data indicate a protective effect by the mass of cellular material on inactivation of cell-associated poliovirus by ozone. While total inactivation of cell-associated poliovirus at 1 NTU was demonstrated at 2.30 mg/L ozone, it should be noted that the initial viral titer of the preparation was lower than any preceeding or subsequent trial. Applied ozone dosages as high as 4.06 mg/L with a residual of 2.56 mg/L after 40 seconds did not result in complete inactivation of 5 NTU suspensions of cell-associated poliovirus. The inactivation data obtained at 1 and 5 NTU for cell-associated poliovirus suggest there was a protection afforded the virus by the presence of cellular material.

This protection may have been provided by competitive oxidation of the associated cellular material with a decreased ozone residual available for inactivation of virus. A comparison of the inactivation obtained at applied ozone dosages of 0.65 mg/L, 2.30 mg/L, and 2.84 mg/L at both 1 and 5 NTU indicates increased poliovirus survival at 5 NTU.

The effect of physical cohesion of the cellular material in the protection of the cell-associated poliovirus was determined by preparing replicate samples, one of which had been sonically treated prior to ozonation in the Sharp apparatus. As seen in Table 18, presonication resulted in increased inactivation in each trial. Concurrent with these experiments, light microscopy displayed definite differences in the physical state of the presonicated samples and the samples not sonically treated both before and after ozonation. A comparison of initial 5 NTU samples of HEp-2 cell-associated

TABLE 17. INACTIVATION OF HEp-2 CELL-ASSOCIATED POLIOVIRUS (SABIN 1) AT 1 AND 5 NTU

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

Turbidity	Initial Ozone (mg/L)	Residual[a] Ozone (mg/L)	Initial Concentration (PFU/ml ozonated stream)	Inactivation (Percent) Time (seconds) 10	20	30
1 NTU	.65	.36	5.3×10^4	99.0	99.2	99.5
	.66	.32	1.2×10^5	97	98	99.0
	.66	.17	2.1×10^5	98	99.4	99.7
	.68	.31	1.5×10^4	96	99.1	99.2
	.76	.30	2.2×10^5	81	93	90
	.87	.28	2.4×10^3	97	99.0	99.6
	2.30	2.30	6.0×10^5	99.5	99.4	100
	2.84	2.09	1.4×10^5	99.7	99.8	99.96
5 NTU	.65	.02	5.1×10^5	97	98	99.3
	1.30	.34	6.5×10^6	97	95	99.3
	2.30	2.30	2.6×10^5	99.92	99.996	99.990
	2.74	1.07	1.4×10^6	99.2	99.5	99.7
	2.84	1.79	8.0×10^3	99.6	99.8	99.7
	3.75	2.85	2.5×10^5	99.6	99.8	99.8
	3.91	3.15	2.0×10^6	99.96	99.997	99.990
	4.06	2.56	1.6×10^6	99.7	99.90	99.96

[a] at 40 seconds

TABLE 18. EFFECT OF PRESONICATION ON INACTIVATION OF HEp-2 CELL-ASSOCIATED POLIOVIRUS (SABIN TYPE 1) AT 1 AND 5 NTU

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

Turbidity	Initial Ozone mg/L	Residual[a] Ozone mg/L	Presonication[b]	Initial Concentration (PFU/ml ozonated stream)	Inactivation (percent) Time (seconds) 10	20	30
1 NTU	.76	.31	-	2.2×10^4	81	93	90
	.76	.39	+	4.8×10^4	97	98	98
5 NTU	3.75	2.85	-	2.5×10^5	99.6	99.8	99.8
	3.75	3.34	+	1.3×10^5	99.90	99.92	99.93
	3.91	3.15	-	2.0×10^6	99.96	99.997	99.990
	3.91	2.78	+	6.6×10^5	99.8	99.97	99.990
	4.06	2.56	-	1.6×10^6	99.7	99.90	99.96
	4.06	3.09	+	1.2×10^6	99.9	99.95	99.990

[a] At 40 seconds

[b] 30 seconds at 50 kcls

poliovirus and a sonically treated replicate of the initial sample of HEp-2 cell-associated poliovirus demonstrates the effect of presonication on the mass of cellular material. The aggregation of the initial sample in Figure 6 was dispersed to single cells as seen in Figure 7 following sonication. The integrity of the initial cells was variable. Figure 6 indicates the presence of both whole and partially fragmented cells in a clump while the presonicated samples in Figure 7 contain only cell fragments.

HEp-2 cell-associated coxsackievirus

The inactivation of HEp-2 associated coxsackieivirus A9 at 1 and 5 NTU is shown in Table 19. Complete inactivation of cell-associated coxsackievirus was not obtained at the highest ozone dosage and contact time possible with the apparatus. In general, 1 NTU preparations exhibited enhanced inactivation in comparison to 5 NTU samples, but total inactivation was not obtained.

The lack of total inactivation concurrent with the enhanced protection exhibited by the NTU cell-associated coxsackievirus suggests that resistance to disinfection by ozone is associated with the presence of the cellular material rather than some inherent property of either coxsackievirus A9 or poliovirus I.

The applied ozone dosages cited in Table 19 represent the maximal dosages obtained from the ozone generator and reaction apparatus previously described. The restricted contact times of the Sharp continuous flow disinfection apparatus did not enable complete inactivation of 1 NTU and 5 NTU samples of HEp-2 cell-associated coxsackievirus or poliovirus.

Batch Reactors

Extended contact times for ozone disinfection were provided in a batch reactor. The results presented in Table 20 indicate that the decrease in ozone concentration did not stabilize with an extended reaction time. Total inactivation of the HEp-2 cell-associated poliovirus was not obtained in the system within the contact time of 75 minutes. The continued survival of HEp-2 cell-associated poliovirus at 75 minutes dictates the necessity for an adequate ozone residual as well as extended contact times. Although initial ozone concentrations of 3.93 mg/L and 2.51 mg/L were used, the remaining ozone residuals at 60 minutes were 0.10 mg/L and 0.11 mg/L, respectively. While the inactivation data obtained at 3.93 mg/L ozone indicate the residual was sufficient to achieve 100 percent inactivation at 75 minutes, at 2.51 mg/L ozone the rate of inactivation was lowered with only 99.1 percent inactivation after 75 minutes. The initial titers of both trials were comparable and would allow for the enhanced survival in either trial.

Continuous Ozonation of HEp-2 Cell-Associated Poliovirus and Coxsackievirus in a Batch Reactor.

Continuous ozonation coupled with extended contact times in a batch reactor resulted in total inactivation of both cell-associated poliovirus

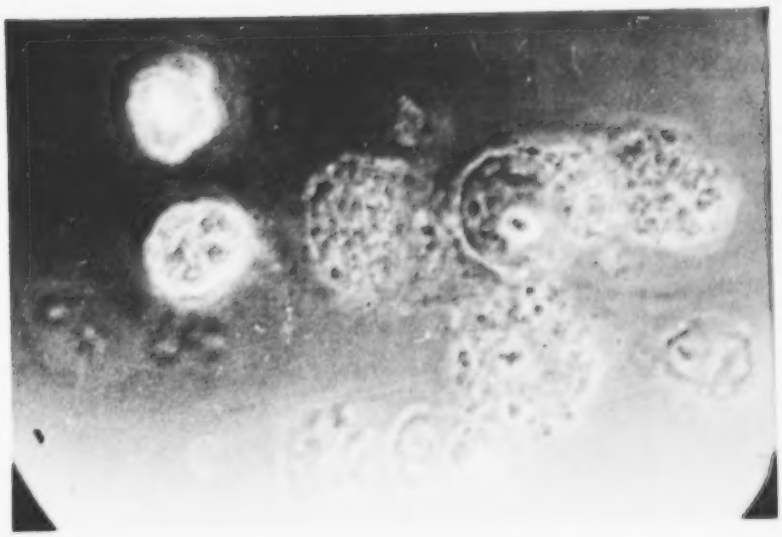

Figure 6. HEp-2 Cells Before Ozonation at 5 NTU

Figure 7. HEp-2 cells sonically treated before ozonation

TABLE 19. INACTIVATION OF HEp-2 CELL-ASSOCIATED COXSACKIEVIRUS (A9) AT 1 AND 5 NTU

pH = 7.0 ± 0.2 Temperature 20°C ± 1°C

Turbidity	Initial Ozone mg/L	Residual[a] Ozone mg/L	Initial Concentration (PFU/ml ozonated stream)	Inactivation (Percent) Time (Seconds) 10	20	30
1 NTU	2.99	1.88	3.7×10^5	99.7	99.93	99.98
	3.59	2.66	9.8×10^4	99.4	99.91	99.97
	3.69	2.93	1.3×10^5	99.92	99.98	99.98
	4.68	2.53	2.4×10^4	99.7	99.92	99.96
5 NTU	2.99	1.85	1.9×10^5	96	92	99.8
	3.59	2.41	1.1×10^6	99.4	99.8	99.7
	3.69	2.93	3.0×10^5	99.0	99.7	99.90
	4.68	2.34	3.7×10^5	99.8	99.98	99.98

[a]At 40 seconds

TABLE 20. BATCH INACTIVATION OF HEp-2 CELL-ASSOCIATED POLIOVIRUS AT 5 NTU

pH = 7.0 ± 0.2 Temperature 20°C ± 1°C

Initial Ozone mg/L	Residual Ozone mg/L Time (minutes) 1	2	5	15	30	60	75	Initial Concentration (PFU/ml stream)	Inactivation (Percent) Time (minutes) 1	2	5	15	30	60	75
3.93	1.56	1.56	1.06	.55	.14	.10	-	1.2×10^5	99.90	99.95	99.994	99.98	99.992	99.997	100
2.51	.86	.76	.64	.34	.20	.11	.17	1.5×10^5	98	99.0	98	99.7	99.4	99.3	99.1

and coxsackievirus as seen in Table 21. This system enabled increased levels of ozone in comparison to that obtained in the Sharp continuous flow system. In conjunction with extended contact times, the smaller volume of the reaction bottle enabled a higher applied dosage of ozone.

Four to five logs of cell-associated poliovirus were inactivated following a 2 minute contact time with initial ozone concentrations of 6.82 and 6.50 mg/L which resulted in ozone residuals of 4.70 and 5.45 mg/L after 2 minutes of reaction time. The HEp-2 cell-associated coxsackievirus exhibited an increased survival time compared to that observed with the cell-associated poliovirus, but at a lower initial ozone concentration and consequent lower residual. In one trial the cell-associated coxsackievirus exhibited survival at 10 minutes with an initial ozone concentration of 5.33 mg/L and a residual of 2.68 mg/L after 10 minutes. However, subsequent trials of 5.19 and 4.81 mg/L initial ozone concentrations with residuals of 3.39 and 2.20 mg/L after 2 minutes showed no survival.

DISCUSSION

The compiled inactivation data obtained from each disinfection system confirm the protective effect provided to virus by association with cellular material. This protection requires very high ozone residuals and extended contact times for disinfection. Some of the current practices use ozone residuals of 0.4 mg/L after five minutes for the disinfection of viruses in drinking water. This investigation has found that such levels are ineffective in the inactivation of HEp-2 cell-associated poliovirus and coxsackievirus.

Interpretation of these findings is subject to qualification by the practical composition of the contaminants. The infected Hep-2 cells utilized in this study were measured at 10 to 15 μm, a size which should dictate their removal by filtration before being subjected to disinfection by ozone in drinking water. Therefore, the initial titers of cell-associated virus contained in a 1 NTU or 5 NTU sample in this investigation would not approximate the number of cell-associated virus present after filtration of drinking water. Nor would the 1 NTU or 5 NTU filtrate subjected to disinfection by ozonation of a heterogenous sample be limited to cell-associated virus. A 1 NTU sample of HEp-2 cell-associated poliovirus with an initial titer of 6.0×10^3 PFU/ml ozonated stream exhibited 100 percent inactivation at an initial ozone concentration of 2.30 mg/L after a 30 second contact time in the Sharp continuous flow apparatus. This finding suggests that with decreased initial titers, as would be present after filtration, total inactivation by ozonation might be obtained. Further experimentation using heterogeneous particulate matter may elucidate the possible use of a disinfectant. However, one must conclude from these data that if one infected cell was present in the heterogeneous particulate material in the filtered water it is possible that virus would survive 0.4 mg/L of ozone after 5 minutes of contact time.

TABLE 21. BATCH INACTIVATION WITH CONTINUOUS OZONATION OF HEp-2 CELL-ASSOCIATED POLIOVIRUS AND COXSACKIEVIRUS AT 5 NTU

pH = 7.0 ± 0.2 Temperature 20°C ± 1°C

Initial ozone mg/L	Residual Ozone mg/L Time (minutes) 1	2	5	10	15	Initial Concentration (PFU/ml ozonated stream)	Inactivation (Percent) Time (minutes) 1	2	5	10	15
						Poliovirus					
6.82	4.70	4.70	4.10	4.45	4.70	2.3×10^5	99.93	100	100	100	100
6.50	4.85	5.45	4.65	4.75	5.05	3.6×10^4	99.90	100	100	100	100
						Coxsackievirus					
5.33	3.76	3.56	3.21	2.68	2.55	1.9×10^4	96	99.7	99.8	99.98	100
5.19	3.78	3.39	2.64	2.90	2.95	7.1×10^4	99.6	99.97	100	100	100
4.81	2.73	2.20	2.18	2.42	2.51	1.9×10^5	99.990	99.996	100	100	100

THE EFFECT OF BENTONITE CLAY ON THE OZONE DISINFECTION OF E. COLI AND VIRUSES IN WATER.

Poliovirus

The results of five poliovirus clay adsorption trials are illustrated in Table 22. Adsorption levels of approximately 10 percent were attained with poliovirus and bentonite clay at pH 7.0. Virus recoveries from the clay and TDW systems averaged 86 to 77 percent, respectively.

Clay particle size used in this work ranged from 3 to 8 μm, a range which is comparable to that of finished water from a filtration plant. These observations were determined with a light microscope.

Complete inactivation of poliovirus was accomplished by using a relatively low ozone dosage, as presented in Table 23. Initial virus concentrations of 8.0×10^1 to 7.4×10^2 PFU/ml, either unadsorbed or at 1 and 5 NTU of bentonite clay, were inactivated within 10 seconds at 0.21 mg/L of ozone. The data indicate that about 0.03 mg/L would have produced complete inactivation of about two logs of adsorbed or unadsorbed virus. Comparison of the unadsorbed virus versus 5 NTU adsorbed trials indicate no or a slight protection against the disinfection of poliovirus when it is adsorbed to bentonite clay.

Coxsackievirus

Adsorption of the Coxsackievirus to the bentonite clay stock averaged 13 percent of the virus added to the clay reactor, as shown in Table 24. A 30 percent increase in virus titer was noted in both the clay and TDW systems.

Disinfection trials with Coxsackievirus produced data similar to those obtained with poliovirus. Table 25 shows the Coxsackievirus results, obtained with an initial virus concentration of 2.0×10^2 to 1.0×10^3 PFU/ml of ozonated stream. At 0.144 mg/L of ozone complete inactivation was achieved within 10 seconds for the unadsorbed, 1 NTU and 5 NTU trials. The data indicate that an initial ozone concentration of about 0.03 mg/L would have produced the same level of inactivation. As with poliovirus, the comparison of the unadsorbed versus 5 NTU adsorbed trials indicates an apparent lack of protection by adsorption of coxsackievirus to bentonite clay. Coxsackievirus adsorption levels at pH 7.0 were only slightly greater than those obtained with poliovirus. No protection at either 1 or 5 NTU of bentonite clay was shown.

f_2 Bacteriophage

Adsorption studies presented in Table 26 indicate that the f_2 virus associated with bentonite clay under the conditions examined. The bentonite-associated f_2 bacteriophage averaged about 1 percent of the clay system phage concentration. Although a low adsorption level, this represented an adsorbed titer range of 2.4×10^3 to 2.2×10^5 PFU/ml of ozonated stream in the disinfection system. Recovery of f_2 from the clay system showed an apparent

TABLE 22. ASSOCIATION OF POLIOVIRUS (SABIN TYPE 1) WITH BENTONITE CLAY

SYSTEM	RANGE OF VIRUS CONCENTRATIONS (PFU/ml)	VIRUS RECOVERY (%)
Virus stock	8.3×10^5 to 1.6×10^6	-
Adsorption to clay $\frac{\text{adsorbed}}{\text{supernatant + adsorbed}} \times 100$	3.0×10^3 to 4.5×10^3	10 (5)[a]
Recovery from clay reactor $\frac{\text{supernatant + adsorbed}}{\text{initial / 25}^b} \times 100$	3.1×10^4 to 5.7×10^4	86 (5)
Recovery from TDW reactor $\frac{\text{control}}{\text{initial / 250}^b} \times 100$	2.3×10^3 to 2.2×10^4	77 (5)

[a] number of trials

[b] dilution of virus stock in the reactor

TABLE 23. INACTIVATION OF POLIOVIRUS (SABIN TYPE 1) UNADSORBED AND ADSORBED AT 1 AND 5 NTU OF BENTONITE CLAY

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Residual[a] Ozone (mg/L)	Initial Concentration (PFU/ml ozonated stream)	Inactivation (%) Time (seconds) 10	20	30
UNADSORBED	0.21	0.20	9.7×10^1	100	100	100
	0.031	0.022	1.3×10^2	100	100	100
	0.016	-	2.3×10^2	92	100	100
	0.015	0.015	8.0×10^1	78	91	100
	0.012	0.011	1.8×10^2	84	81	96
	0.007	0.007	7.4×10^2	77	85	87
1 NTU ADSORBED	0.21	0.20	1.0×10^2	100	100	100
			1.3×10^2	100	100	100
5 NTU ADSORBED	0.21	-	1.0×10^2	98	100	100
	0.031	-	1.4×10^2	93	100	100
	0.016	-	1.1×10^2	59	78	75
	0.015	-	1.5×10^2	57	81	97
	0.012	-	1.1×10^2	81	90	96
	0.007	-				

[a] taken at 40 seconds

TABLE 24. ASSOCIATION OF COXSACKIEVIEUS A9 WITH BENTONITE CLAY

SYSTEM	RANGE OF VIRUS CONCENTRATIONS (PFU/ml)	VIRUS RECOVERY (%)
Virus stock	1.5×10^6 to 4.7×10^6	-
Adsorption to clay $\frac{\text{adsorbed}}{\text{supernatant + adsorbed}} \times 100$	6.4×10^3 to 2.9×10^4	13 (7)[a]
Recovery from clay reactor $\frac{\text{supernatant + adsorbed}}{\text{initial / 25}^b} \times 100$	4.6×10^4 to 2.1×10^5	130 (3)
Recovery from TDW reactor $\frac{\text{control}}{\text{initial / 250}^b} \times 100$	6.3×10^3 to 2.3×10^4	130 (3)

[a] number of trials

[b] dilution of virus stock in the reactor

TABLE 25. INACTIVATION OF COXSACKIEVIRUS A9 UNADSORBED AND ADSORBED AT 1 AND 5 NTU OF BENTONITE CLAY

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Residual[a] Ozone (mg/L)	Initial Concentration (PFU/ml ozonated stream)	Inactivation (%) Time (seconds)		
				10	20	30
UNADSORBED	0.144	0.142	2.2×10^2	100	100	100
	0.040	0.030	2.2×10^2	100	100	100
	0.020	0.016	1.0×10^3	100	100	100
	0.019	0.018	3.9×10^2	95	100	100
	0.016	0.016	2.8×10^2	46	64	80
	0.014	0.012	3.7×10^2	24	62	76
1 NTU ADSORBED	0.144	0.134	2.0×10^2	100	100	100
5 NTU ADSORBED	0.144	0.137	2.8×10^2	100	100	100
	0.040	-	5.2×10^2	100	100	100
	0.020	-	8.0×10^2	99.6	100	100
	0.019	-	2.2×10^2	97	100	100
	0.016	-	4.1×10^2	49	71	91
	0.014	-	3.5×10^2	26	51	84

[a]Taken at 40 seconds

TABLE 26. ASSOCIATION OF f_2 BACTERIOPHAGE WITH BENTONITE CLAY

SYSTEM	RANGE OF VIRUS CONCENTRATIONS (PFU/ml)	VIRUS RECOVERY (%)
Virus stock	8.8×10^8 to 2.3×10^9	-
Adsorption to clay $\frac{\text{adsorbed}}{\text{supernatant + adsorbed}} \times 100$	2.8×10^5 to 3.9×10^6	1 (13)[a]
Recovery from clay reactor $\frac{\text{supernatant + adsorbed}}{\text{initial / 25}^b} \times 100$	1.6×10^7 to 3.3×10^8	173 (8)

[a] Number of trials

[b] Dilution of virus stock in reactor

73 percent increase in virus concentration. This substantial increase in titer shown over many trials suggests a breakup of f_2 aggregates during the adsorption procedure. Of the two clay system components (supernatant and adsorbed) the phage increase was noted in the supernatant fraction. Floyd and Sharp (38) have investigated the aggregation of poliovirus and reovirus caused by dilution in water and by varying conditions of salinity and pH. They reported that aggregation may be a function of virus concentration, pH, or the presence of particular ions in solution. Throughout the f_2 virus experimentation, salt concentration (0.01 M NaCl) and pH (7.0) remain constant. Bentonite clay contains exchangable, surface bound cations which may have influenced a break up of aggregrates. Unfortunately, inconclusive data were obtained with the TDW control, so it is uncertain whether the presence of bentonite clay was responsible for the increase.

Inactivation data for the f_2 phage are shown in Table 27. Initial ozone concentrations of 0.25 to 0.50 mg/L resulted in complete inactivation of unadsorbed phage in concentrations of 2.6×10^3 to 2.2×10^5 PFU/ml within 10 seconds. Survival was noted at 10 seconds when the ozone level was reduced to 0.061 mg/L, however, 30 seconds exposure to that concentration was sufficient to completely inactivate the phage.

The inactivation levels of f_2 associated with bentonite were less than those determined in the unadsorbed system. In the presence of the clay, initial virus concentrations of 2.4×10^3 to 2.2×10^5 PFU/ml consistently showed survival when exposed for 10 seconds to 0.25 to 0.50 mg/L of ozone with about the same residual ozone concentrations. Further, a 2.2 mg/L initial ozone concentration was insufficient to completely inactivate the phage within 10 seconds. From these results it appears that contact time is more significant than ozone concentration as a parameter in the disinfection of adsorbed f_2 bacteriophage. Protection of the phage by adsorption is also suggested.

Escherichia coli

The association of Escherichia coli with bentonite was not quantitatively determined. Separation of the bentonite-associated and freely suspended E. coli was hindered by the relative size of the two components. The dimensions of E. coli (0.3 x 1.5 μm) as compared to the clay particles (3 to 8 μm) indicate that association from other than a surface phenomenon would have been unlikely.

As presented in Table 28, similar inactivation data were obtained for the unadsorbed versus bentonite-associated E. coli. In both cases, initial bacteria concentrations of 9.4×10^7 to 3.6×10^8 CFU/ 100 ml were reduced at least three logs within 10 seconds exposure to ozone dosage of 0.038 to 0.53 mg/L. Complete inactivation was not achieved within 10 seconds, but was consistently complete within 30 seconds over the range of ozone dosages studies. Disinfection trials conducted with the unadsorbed E. coli versus E. coli adsorbed to 5 NTU of bentonite indicate a lack of protection by the clay. Here, as in the f_2 data, contact time apears to be the dominant disinfection parameter.

TABLE 27. INACTIVATION OF f_2 BACTERIOPHAGE UNADSORBED AND ADSORBED AT 1 AND 5 NTU OF BENTONITE CLAY

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Residual[a] Ozone (mg/L)	Initial Concentration (PFU/ml ozonated stream)	Inactivation (%) Time (seconds) 10	20	30
UNADSORBED	0.50	0.39	2.6×10^5	100	100	100
	0.48	0.35	1.2×10^5	100	100	100
	0.47	0.46	2.1×10^4	100	100	100
	0.45	-	7.3×10^5	100	100	100
	0.26	-	1.3×10^5	100	100	100
	0.25	0.20	2.2×10^4	100	100	100
	0.061	0.045	1.7×10^4	99.9	99.96	100
1 NTU ADSORBED	2.2	-	7.1×10^4	99.97	100	100
	0.46	-	1.7×10^4	99.98	100	-
	0.45	0.37	1.9×10^4	99.997	99.98	100
	0.26	-	3.0×10^4	99.8	100	100
	0.25	-	5.7×10^4	99.995	99.98	100
5 NTU ADSORBED	2.2	-	2.0×10^5	99.97	100	100
	0.50	0.36	2.4×10^3	96	99.6	100
	0.48	-	7.7×10^4	99.991	99.999	99.99
	0.47	0.36	6.9×10^4	99.6	99.97	99.98
	0.46	-	2.8×10^4	99.4	100	100
	0.45	0.26	3.0×10^4	97	99.3	100
	0.45	0.45	1.8×10^4	99.97	99.997	99.97
	0.26	-	5.7×10^5	99.99	99.990	99.999
	0.25	0.25	2.2×10^4	99.8	99.996	99.997
	0.061	-	1.5×10^4	99.9	99.95	100

a Taken at 40 seconds

TABLE 28. INACTIVATION OF *ESCHERICHIA COLI* UNADSORBED AND IN ASSOCIATION WITH 5 NTU OF BENTONITE CLAY

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

	Initial Ozone (mg/L)	Residual[a] Ozone (mg/L)	Initial Concentration CFU/100 ml ozonated stream)	Inactivation (%) Time (seconds) 10	20	30
UNADSORBED	0.53	0.49	2.0×10^8	99.9998	100	100
	0.20	0.17	1.4×10^8	99.90	99.998	100
	0.071	0.026	3.0×10^8	99.999	99.9992	100
	0.062	0.034	3.1×10^8	99.998	100	100
	0.055	0.014	1.1×10^8	99.9990	100	100
	0.038	-	3.6×10^8	99.9990	100	100
ASSOCIATED WITH 5 TU OF BENTONITE	0.53	0.53	1.8×10^8	99.9998	100	100
	0.20	0.16	1.3×10^8	99.9995	100	100
	0.071	-	1.6×10^8	99.98	99.993	100
	0.062	-	2.1×10^7	-	100	100
	0.055	-	9.4×10^8	99.994	100	100
	0.038	-	1.8×10^8	99.997	100	100

[a]Taken at 40 seconds

DISCUSSION

The virus adsorption levels obtained throughout this research were significantly lower than those reported by Boardman and Sproul (29), whose procedures were utilized with modifications.

Stagg, et al.(39) reported that association of MS-2 bacteriophage with bentonite clay was influenced by the titer of unadsorbed viruses, clay concentration, cation concentration, temperature, stirring rate, and the presence of soluble organics. While our experiments were devised to minimize the above variables, some uncertainties remain, such as the use of a bentonite suspension as an adsorbant which had been subjected to preozonation. Nevertheless, regardless of percentage, the adsorption levels were consistent for each organism studied over numerous trials.

A detailed review of research relating turbidity to disinfection was compiled by Hoff (17). Included in the report were studies of the effects of inorganic and organic turbidity on disinfection of poliovirus type 1 with hypochlorous acid (HOCl). The results showed that virus adsorbed to bentonite exhibited inactivation rates similar to, or perhaps slightly slower than, freely suspended virus. Similar first order inactivation curves were generated with the adsorbed and unadsorbed poliovirus. Conversely, organic turbidity represented by cell debris had a pronounced protective effect on associated virus. Inactivation rates slowed as exposure time increased, indicating the existence of various degrees of protection and some extremely resistant complexes. The report concluded that turbidity is not an ideal measure of the disinfectability of water since interference with disinfection depends more on the types of turbidity present than the number of turbidity units.

The results obtained in this study using poliovirus and coxsackievirus tend to support the above findings. Comparison of adsorbed versus unadsorbed inactivation data reveals the lack of protection offered the virus by the inorganic clay particles. Evidently no physical interference was provided by the bentonite, nor would chemical reactivity between the ozone and the demand free clay particles be anticipated. In addition, due to the sensitivity of the adsorbed virus to low concentrations of ozone, it appears that ozone diffuses easily to the clay particles, inactivating the adsorbed virus at a rate that is approximately the inactivation rate for unadsorbed virus.

Microorganisms have been found to associate with clay particles of a size that may escape conventional filtration plants and enter potable water supplies. Poliovirus (Sabin type 1), coxsackievirus A9, f_2 bacteriophage, and Escherichia coli associated with bentonite were inactivated by ozone at rates similar to or, as is believed the case with f_2, at slightly slower rates than freely suspended organisms.

A comparison of various inactivation data for unadsorbed organisms used in this study is shown in Table 29. In selecting the data an attempt was made to include only those where some survival was noted after 30 seconds of contact and where the residual was measured after 40 seconds. While the

TABLE 29. SUMMARY OF INACTIVATION OF UNADSORBED ORGANISMS

pH = 7.0 ± 0.2 Temperature = 20°C ± 1°C

Organism	Initial Ozone mg/L	Residual Ozone (40 seconds) mg/L	Inactivation in 30 Seconds CFU/100ml or PFU/ml	Text Reference Table No.
S. fecalis	0.13	NM	1.5×10^9	6
E. coli	0.014	NM	9.9×10^7	16
	0.017	0.014	5.2×10^7	16
	0.04	0.029	7.7×10^8	16
	0.055	0.014	1.1×10^6 +	28
	0.096	0.067	1.1×10^{10} +	7
	0.38	NM	1.1×10^8	6
	0.56	NM	1.9×10^7	8
	0.58	0.27	2.4×10^7	8
Poliovirus 1	0.012	0.008	1.5×10^4	10
	0.012	0.011	1.7×10^2	23
	0.015	0.011	1.4×10^4	10
	0.031	0.022	1.3×10^2 +	23
	0.085	0.067	2.4×10^3 +	10
Coxsackie A9	0.012	0.010	9.1×10^3 +	12
	0.014	0.012	2.8×10^2	25
	0.016	0.016	2.2×10^2	25
	0.032	0.027	9.1×10^3	12
Porcine picornavirus 3	0.036	0.018	2.2×10^4	5
f_2	0.115	0.083	7.0×10^6	14
	0.25	0.020	2.2×10^5 +	27

NM=not measured

data do not lend themselves to easy comparison it appears that the order of increasing resistance to disinfection was E. coli, poliovirus 1 and Coxsackievirus A9. This ranking was based on comparing E. coli, polio and Coxsackie at 0.017 mg/L, 0.015 mg/L and 0.016 mg/L ozone at the start of the experiment. The corresponding inactivations obtained were 5.2×10^7 CFU/100 ml, 1.4×10^4 PFU/ml, and 2.2×10^2 PFU/ml in 30 seconds.

OTHER REPORTS BASED ON THIS RESEARCH

Additional published material, based on research conducted under this grant includes the following:

Theses

1. Howser, Diana M. The Effect of Fecal Material on Ozone Disinfection of Coliform Bacteria and Enteric Viruses in Water.

2. Walsh, Douglas S. The Effect of Hydrated Aluminum Oxide Floc on Ozone Disinfection of Bacteria and Viruses in Water.

3. Boyce, Douglas S. The Effect of Bentonite Clay on Ozone Disinfection of Bacteria and Viruses in Water.

Copies can be obtained from:

Fogle Library
University of Maine
Orono, Maine 04473

Publications

1. Sproul, O.J., M.A. Emerson, D.M. Howser, D.S. Boyce, D.S. Walsh and C.D. Buck. "Effect of Particulate Matter on Virus Inactivation by Ozone." Proceedings Annual Meeting American Water Works Association, 1978 (presented at Annual Meeting, June, 1978).

2. Howser, D.M., M.A. Emerson, D.S. Walsh, O.J. Sproul and C.E. Buck. "Ozone Inactivation in Cell and Fecal Associated Bacteria and Virus." Presented at Water Pollution Control Federation Anaheim Conference, Cnaheim, California, October 1978. (submitted to JWPCF for publication).

Papers in Preparation

1. Sproul, O.J., M.A. Emerson, D.M. Howser, D.S. Boyce, D.S. Walsh and C.E. Buck. Effect of Particulate Matter on Virus Inactivation by Ozone.

2. Emerson, M.A., O.J. Sproul and C.E. Buck. Ozone Inactivation of HEp2 Cell-Associated poliovirus and Coxsackievirus.

3. Walsh, D.S., O.J. Sproul and C.E. Buck. Ozone Disinfection of Bacteria and Viruses Associated with Hydrated Aluminum Oxide Floc.

4. Boyce, D.S., O.J. Sproul and C.E. Buck. Ozone Disinfection of Bacteria and Viruses Associated with Bentonite Clay.

LITERATURE CITED

1. Nupen, E.M., "Viruses in Renovated Waters", Viruses in Water, Berg, G., et al., ed., American Public Health Association, Inc. Washington, D.C. (1976).

2. Long, W.N. and Bell, F.A., Jr., "Health Factors and Reused Waters", American Water Works Association 64 (4): 220 (1972).

3. Craun, G.F., McCabe, L.J. and Hughes, J.M., "Waterborne Disease Outbreaks in the U.S. --1971-1974", J. Am. Water Works Assn. 68 (8): 420 (1976).

4. "Engineering Evaluation of Virus Hazard in Water:, Committee on Environmental Quality Management, Berger, B.B., Chmn., Jour. San. Eng. Div., ASCE, 96 (Sal): 111 (1970).

5. Geldreich, E.E., "Origins of Microbial Pollution in Streams", Transmission of Viruses by the Water Route, Berg, G., ed., John Wiley and Sons, New York (1967).

6. Kelly, S. and Sanderson, W.W., "Density of Enteroviruses in Sewage", J. Water Pollution Control Fed. 32, 1269 (1960).

7. Cookson, J.T., Jr., "Virus and Water Supply", J. Amer. Water Works Assn. 66 (12): 707 (1974).

8. Berg, G., "An Integrated Approach to the Problem of Viruses in Water:, Proceedings of the National Specialty Conference on Disinfection, July 1970. ASCE, New York, New York (1971).

9. Gerba, C.P., Wallis, C. and Melnick, J.L., "Viruses in Water: The Problem, Some Solutions", Environmental Science and Technology 9 (13): 1122 (1975).

10. Clark, N.A., Berg, G., Kabler, P.W. and Chang, S.L., "Human Enteric Viruses in Water: Source Survival and Removability", Advances in Water Pollution Research, Vol. 2, MacMillan Company, New York (1962).

11. Metcalf, T.G., "Indicators for Viruses in Natural Waters", Water Pollution Microbiology, Vol, 2, Mitchell, R., ed., John Wiley and Sons, New York (1978).

12. Sproul, O.J., "Removal of Viruses by Treatment Processes", Viruses in Water, Berg, G., et al., ed., American Public Health Association, Inc., Washington, D.C. (1976).

13. Plotkins, S.A. and Katz, M., "The Minimal Infective Dose", Transmission of Viruses by the Water Route, Berg, G., ed., John Wiley and Sons, New York (1967).

14. Berg, G., "Reassessment of the Virus Problem in Sewage and in Surface and Renovated Waters", Progress in Water Technology, Jenkins, S.H., ed., Pergammon Press, New York (1973).

15. Schaub, S.A. and Sorber, C.A., "Viruses on Solids in Water", Viruses in Water, Berg, G., et al., ed., American Public Health Association, Inc., Washington, D.C. (1976).

16. Symons, J.M. and Hoff, J.C., "Rationale for Turbidity Maximum Contaminant Level", Proceedings AWWA Water Quality Technology Conference, Atlanta, Georgia (1975).

17. Hoff, J.C., "The Relationship of Turbidity to Disinfection of Potable Water", Evaluation of the Microbiology Standards for Drinking Water, Hendricks, C., ed., EPA-570/9-78-00C (1978).

18. "Ozone, Chlorine Dioxide, and Chloramines as Alternatives to Chlorine for Disinfection of Drinking Water: State-of-the-Art", Symons, J.M., ed., Water Supply Research, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio (1977).

19. Page, T., Harris, R.H., and Epstein, S.S., "Drinking Water and Cancer Mortality in Louisiana", Science 193, 55 (1976).

20. Kuzma, R.J., Kuzma, D.M. and Buncher, C.R., "Ohio Drinking Water Source and Cancer Rates", Am. J. of Public Health 67 (8): 725 (1977).

21. Majumdar, S.B. and Sproul, O.J., "Technical and Economic Aspects of Water and Wastewater Ozonation: A Critical Review", Water Res. 8, 253 (1974).

22. Venosa, A.D., "Ozone as a Water and Wastewater Disinfectant: A literature Review", Ozone in Water and Wastewater Treatment, Evans, F.L., ed., Ann Arbor Science Publishers, Inc., Michigan (1972).

23. Lawrence, J. and Cappelli, F.P., "Ozone in Drinking Water Treatment: A Review", The Science of the Total Environment 7 (2): 99 (1977).

24. Watson, J.T., and Drewry, W.A., "Adsorption of f_2 Bacteriophage by Activated Carbon and Ion Exchange Resins", Civil Engineering, The University of Tennessee, Research Series No. 11, 23 (1971).

25. Cooper, S. and Zinder, N.D., "The Growth of RNA Bacteriophage: The Role of DNA Synthesis", Virology 18, 405 (1962).

26. Gentile, D.M., Unpublished Procedure (1975).

27. Standard Methods for the Examination of Water and Wastewater, 14th ed., American Public Health Assoc. Inc., Washington, D.C. (1975).

28. Guskey, L.E. and Wolff, D.A., "Concentration and Purification of Poliovirus by Ultrafiltration and Isopycnic Centrifugation", Appl, Microbiology 24 (1): 13 (1972).

29. Boardman, G.D. and Sproul, O.J., "Adsorption as a Protective Mechanism for Waterborne Viruses", Completion Report, Project A-030-ME, Land and Water Resources Institute, University of Maine, Orono (1977).

30. Wolf, K. and Quimby, M.C., "Fish Virology: Procedures and Preparation of Materials for Plaquing Fish Viruses in Normal Atmosphere", United States Department of the Interior, US ISSN 0071-6493, FDL-35 (1973).

31. Karber, G., "Beitrag zur Kollektiven Behandlung Pharmakologischer Reihenversuche", Arch. Exptl. Path. Pharmakol., 162, 480 (1931).

32. Adams, M.H., Bacteriophage, Interscience Publishers, New York (1969).

33. Shechter, H., "Spectrophotometric Method for Determination of Ozone in Aqueous Solutions", Water Research 7, 729 (1973).

34. Tate, C.H., Lang, J.S. and Hutchinson, H.L., "Pilot Plant Tests of Direct Filtration", J. Am. Water Works Assn. 69 (7): 343 (1977).

35. Rickert, D.A. and Hunter, J.V., "Rapid Fractionation and Materials Balance of Solids Fractions in Wastewater and Wastewater Effluent", Jour. Water Poll. Cont. Fed. 39 (9): 1475 (1967).

36. Ward, R.L. and Ashley, C.S., "Identification of the Vircidal Agent in Wastewater Sludge", Appl. Environ. Microbiol. 33, 860 (1977).

37. Diaper, E.W.J., "Disinfection of Water and Wastewater using Ozone", Disinfection Water and Wastewater, Johnson, D.J., ed., Ann Arbor Science Publishers, Inc., Michigan (1975).

38. Floyd, R. and Sharp, D.G., "Aggregation of Poliovirus and Reovirus by Dilution in Water", Appl, Environ. Microbiol. 35 (6): 1084 (1978).

39. Stagg, C.H., Wallis, C., and Ward, C.H., "Inactivation of Clay Associated Bacteriophage MS-2 by Chlorine", Appl, Environ. Microbiol. 33 (2): 385 (1977).